

**Pik1p, a phosphatidylinositol 4-kinase, interacts with Cdc4p,
a contractile ring protein essential for cytokinesis in fission
yeast**

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in partial fulfillment of the Requirements

For the Degree of

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in the

Department of Microbiology and Immunology

University of Saskatchewan

by

Sarah Katherina Steinbach

Dedicated to my parents.

**University of Saskatchewan
Permission to use Postgraduate Thesis**

TITLE OF THESIS	Pik1p, a phosphatidylinositol 4-kinase, interacts with Cdc4p, a contractile ring protein essential for cytokinesis in fission yeast
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DEPARTMENT	Microbiology and Immunology
DEGREE	Doctor of Philosophy

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Abstract

A yeast two-hybrid assay suggested the possibility of an interaction between Cdc4p, a small EF-hand protein essential for cytokinesis, and Pik1p in *S. pombe*. This interaction was unexpected, as one function of Cdc4p is that of an essential light chain, bound to the first IQ-motif of type II myosins, whereas Pik1p is a phosphatidylinositol 4-kinase. The objective of this work was to analyze the effects of Pik1p lipid kinase activity on the cell cycle of *S. pombe*. Another goal of this study was to evaluate the functional significance of the interaction between Cdc4p and Pik1p. This was performed by generating two mutants of *pik1*: one that abolished lipid kinase activity (*pik1*^{D709A}) and one that abolished Pik1p Cdc4p-binding activity (*pik1*^{R838A}). Pik1p has a conserved IQ-motif in its C-terminal region. A mutation in this site (R838A), homologous to a residue which was mutated in myosin and abrogated the interaction with Cdc4p, prevented the interaction with Cdc4p in a yeast two-hybrid assay and ELISA. An increase in lipid kinase activity was observed in cell extracts upon ectopic expression of *pik1*^{wt} from an episome, which was abolished by a mutation in the lipid kinase domain of Pik1p (D709A), but not by the R838A mutation. However, little to no increase in lipid kinase activity was observed upon ectopic expression of *pik1*^{wt} and *pik1*^{R838A} in a strain carrying a conditionally lethal allele of *cdc4* (*cdc4*^{G107S}). This mutation in Cdc4p was shown previously to prevent the interaction with Pik1p in yeast two-hybrid assays. Ectopic expression of *pik1*^{wt} suppressed cell proliferation, with disruption of actin cytoskeletal structures and contractile ring formation. These results were not observed with the ectopic expression of the *pik1*^{R838A} mutant or when *pik1*^{wt} was expressed in the *cdc4*^{G107S} strain. Ectopic expression of *pik1*^{R838A} resulted in cell

shortening, likely through inhibition of growth, and many of the short cells showed an accumulation of the expressed Pik1p protein at the cell tips. Formation of the contractile ring appeared unaffected in cells with ectopic expression of the *pik1^{D709A}* mutant, but many of these cells had thick or more than one septum, characteristic of a septation defect. The ectopic expression phenotypes were dosage dependent since lower levels of expression greatly reduced the severity of the ectopic phenotypes. Pik1p lipid kinase activity is essential and, based on ectopic expression studies, is required for septation. There is a physical and functional interaction between Cdc4p and Pik1p which is not essential for cell viability, but suggests a role for Cdc4p in phosphoinositide metabolism.

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List of Abbreviations

3-AT	3-amino-1',2',4'-triazole
Ade	adenine
AP	adaptor protein
APC	anaphase promoting complex
ASR	arrest of secretion
ATP	adenosine triphosphate
BFA	brefeldin A
bp	base-pair
cDNA	complementary DNA
CF	calcofluor white
DAPI	4', 6-diamidino-2-phenylindole
DPM	disintegrations per minute
DYRK	dual specificity tyrosine phosphorylation regulated kinase
ELC	essential light chain
ER	endoplasmic reticulum
FAPP	four-phosphate adaptor protein
FITC	fluorescein isothiocyanate
<i>g</i>	gravity (9.8 kg.m.s ⁻²)
G1	gap1
G2	gap2
GAT	GGA and Tom1p
GDP	guanosine diphosphate

GFP	green fluorescent protein
GRAM	glucosyltransferases, Rab-like GTPase activators and myotubularins
GTP	guanine triphosphate
HA	haemagglutinin
IQGAP	IQ containing GTPase activating protein
kb	kilo base-pair
kDa	kilo Daltons
Leu	leucine
LKU	lipid kinase unique
MIPA	micropexophagy apparatus
Mr	molecular weight
N ₂	nitrogen gas
NES	nuclear export signal
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
PAS	preautophagosomal structure
PBS	phosphate buffered saline
PCH	pombe Cdc15p homology
PCR	polymerase chain reaction
PH	pleckstrin homology
PITP	phosphatidylinositol transfer protein
PtdIns	phosphatidylinositol
RE	recycling endosome

RLC	regulatory light chain
RT	reverse transcriptase
SAC	spindle assembly checkpoint
SD	synthetic dextrose
SDS	sodium dodecyl sulfate
SIN	septation initiation network
SOC	spindle orientation checkpoint
SPB	spindle pole body
TLC	thin layer chromatography
TGN	<i>trans</i> - Golgi network
TRAPP	transport protein particle
Trp	tryptophan
ts	temperature sensitive
SSC	sodium chloride and sodium citrate
TAE	Tris-acetate-EDTA buffer
Ura	uracil
VSVG	vesicular stomatitis virus G-protein
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactoside

List of Materials and Supplies

Product	Company name	Origin	Catalogue Number
3-AT	Sigma	New York, NY	T-3383
Acrylamide	BDH	Canada	44313
Agar	Difco	USA	214530
Adenine	Sigma	New York, NY	A-8626
Ammonium persulfate	Sigma	New York, NY	A-9164
Ampicillin	Sigma	New York, NY	A9518
Aquasol	Perkin Elmer	Waltham, MA	6NE9349
ATP	Amersham	Piscataway, NJ	27-2056-01
<i>Bam</i> HI	NEB	Ipswich, MA	R0136S
<i>Bgl</i> II	NEB	Ipswich, MA	R0144S
Bisacrylamide	BDH	Canada	44300
β -mercaptoethanol	Sigma	New York, NY	M7154
Bottle top filter	Nalgene	New York, NY	291-4545
Bradford reagent	Sigma	New York, NY	B-6916
BSA- Bovine serum albumin	Sigma	New York, NY	A-4503
CaCl ₂	BDH	Canada	B10070
Calcofluor white	Sigma	New York, NY	F3543-1G
cDNA synthesis kit	Amersham	Piscataway, NJ	27-9261-01
chloramphenicol	Sigma	New York, NY	C-0378

<i>Clal</i>	NEB	Ipswich, MA	R0197S
Cryovials	Nalgene	New York, NY	5000-1020
DAPI	Sigma	New York, NY	D-9542
dNTPs	Invitrogen	USA	10297-018
ECL kit	Amersham	Piscataway, NJ	RPN2132
<i>EcoRI</i>	NEB	Ipswich, MA	R0101S
EDTA- Ethylene diamine tetraacetic acid	Fisher	Ottawa, ON	E-478
EMM- Edinburgh minimal medium	QBiogene	Irvine, CA	4110-032
Eppendorf 1.5 mL tubes	Brinkmann	New York, NY	22-36411-1
Ethanol	Brenntag Canada	Saskatoon, SK	UN1170
Ethidium bromide	Sigma	New York, NY	E-8751
FITC-phalloidin	Sigma	New York, NY	P-5157
Glacial acetic acid	Fisher	Ottawa, ON	A38P4
Glucose	Sigma	New York, NY	G-8270
Glycerol	Anachemia	Mississauga, ON	43567-360
Glycine	Fisher	Ottawa, ON	G46-1
Goat α -rabbit IgG-HRP	Sigma	New York, NY	A-9169
Goat α -rabbit IgG Texas Red conjugate	Santa Cruz	Santa Cruz, CA	SC-2780
H ₂ SO ₄	Fisher	Ottawa, ON	A298-212

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Fisher	Ottawa, ON	BP310-500
Histidine	Sigma	New York, NY	H-8125
Iodine crystals	Sigma	New York, NY	I-3380-50G
Isopropyl alcohol	EM	New York, NY	PX 1834-1
Kanamycin	Boehringer Mannheim	Laval, QC	84144821-48
KCl	Fisher	Ottawa, ON	P217-500
KH ₂ PO ₄	BDH	Canada	ACS657
Kodak BioMax XAR Film	Kodak	New Haven, CT	81555918
Leucine	Sigma	New York, NY	L-8000
Lithium Acetate	Sigma	New York, NY	L-4158
Lysine	Sigma	New York, NY	L-5626
Lysing enzyme	Sigma	New York, NY	L-2265
ME- Malt Extract	QBiogene	Irvine, CA	4103-012
Methanol	Univar Canada Ltd.	Saskatoon, SK	UN1230
MgCl ₂	Fisher	Ottawa, ON	M33B
MgSO ₄	Fisher	Ottawa, ON	M63-500
Multiwall (96) plates	Falcon	New York, NY	353912
NaCl ₂	EMD	San Diego, CA	SX0420-3
Na ₂ HPO ₄	EMD	New York, NY	SX0720-1

<i>Nde</i> I	NEB	Ipswich, MA	R0111S
Nitrocellulose membrane	Perkin Elmer	Waltham, MA	NBA083B
<i>Not</i> I	NEB	Ipswich, MA	RO189S
Novozyme 234	Novo Industri	Bagsvaerd Denmark	N3457
³² P-γ-ATP	Amersham	Piscataway, NJ	AA0018
Parafilm	VWR	Mississauga, ON	52858-076
Paraformaldehyde	Sigma	New York, NY	P-6148
pBI771	PBI	Saskatoon, SK	By request
pBI880	PBI	Saskatoon, SK	By request
pBluescript KS(-)	Stratagene	La Jolla, CA	212208
PEG- Polyethylene glycol 3500	Sigma	New York, NY	P-3640
PEG- Polyethylene glycol 8000	Fisher	Ottawa, ON	BP233-1
pGEM-T Easy	Promega	New York, NY	A1360
Phenol/chloroform/ isoamyl alcohol	Sigma	New York, NY	P-2069
Phenylenediamine	Sigma	New York, NY	P-1519
Phloxin B	Sigma	New York, NY	P-4030
Plastic wrap	Saran	Saskatoon, SK	Stores
Polypropylene round bottom tubes	Falcon	New York, NY	352059

pRSETB	Invitrogen	Carlsbad, CA	V351-20
PtdIns	Avanti	Alabaster, AL	840042
PtdIns4P	Avanti	Alabaster, AL	840045X
PtdIns(4,5)P ₂	Avanti	Alabaster, AL	840046X
PtdIns(3,4,5)P ₃	Avanti	Alabaster, AL	850186X
Poly-L-lysine	Sigma	New York, NY	P-4707
pREP1/41/81	ATCC	Manassas, VA	87685
Protease inhibitor cocktail for yeast	Sigma	New York, NY	P-8215
Protease inhibitor cocktail for bacteria	Sigma	New York, NY	P-8465
Protein A-sepharose	Sigma	New York, NY	P-3391
Qiagen gel purification kit	Qiagen	Mississauga, ON	28106
Qiagen maxiprep kit	Qiagen	Mississauga, ON	12162
Salmon Sperm Carrier DNA	Sigma	New York, NY	D-1626
<i>SalI</i>	NEB	Ipswich, MA	R0138S
SDS	Gibco	New York, NY	15525-017
Silica gel 60 TLC plates	VWR	Mississauga, ON	WC4860-820
Sodium Acetate	Fisher	Ottawa, ON	S210B
Sodium Azide (NaN ₃)	BDH	New York, NY	87980
Sodium Hydroxide	Fisher	Ottawa, ON	S318B

(NaOH)			
Sorbitol	Fisher	Ottawa, ON	S459-500
<i>SpeI</i>	NEB	Ipswich, MA	R0133S
SSC	Fisher	Ottawa, ON	S279B-500
T4 DNA Ligase	NEB	Ipswich, MA	M0202S
T4 polynucleotide kinase (PNK)	Amersham	Piscataway, NJ	70031Y
T7 DNA polymerase	Amersham	Piscataway, NJ	70017Y
<i>Taq</i>	Invitrogen	USA	10342-020
TEMED	Sigma	New York, NY	T-9281
Tetracycline	Sigma	New York, NY	T-3383
Thiamine	Sigma	New York, NY	T-1270
TMB	Sigma	New York, NY	T-8768
Tris	Roche	New York, NY	03118142001
Tris-phenol (pH 7.5-8.0)	Sigma	New York, NY	P-4557
Triton-X	Sigma	New York, NY	T-6878
Trizol	Invitrogen	USA	15596-018
Tryptophan	Sigma	New York, NY	T-0254
Tryptone	BDH	Canada	211705
Tween 20	Sigma	New York, NY	P-7949
Uracil	Sigma	New York, NY	U-0750
Yeast extract	Difco	USA	212750

YES	QBiogene	Irvine, CA	4101-532
X-gal	MBI Fermentas	New York, NY	R0402
Zymolase 20T	ICN	Ohio, USA	320921

Chapter 1: Literature review

1.1. *Schizosaccharomyces pombe*

S. pombe was first identified as a good model for cell cycle research by Murdoch Mitchison, professor of Zoology at the University of Edinburgh. He reported that he discovered *S. pombe* almost serendipitously while flipping through a book on yeast taxonomy by Lodder and Kreger van Rij published in 1952 (Mitchison, 1957). Mitchison thought *S. pombe* was attractive for cell cycle studies because it was symmetrical, and divided by medial fission in a manner similar to that of animal cells, unlike *Saccharomyces cerevisiae* which divides by budding (Mitchison, 1957). It was later discovered that *S. pombe* also has minimal gene duplication whereby only one or two genes encode components of cellular processes. This is advantageous because mechanistically, *S. pombe* provides a more basic view of molecular processes compared to multicellular organisms. Indeed, many other eukaryotes, including metazoans, have multiple copies of many genes. Working with *S. pombe* is becoming increasingly easy with the availability of the full genomic sequence and well established molecular genetics and cell biology techniques. Furthermore, high throughput genomic studies are

also underway and/or have already been performed, including global gene expression profiles for virtually every gene in the fission yeast genome, and the large scale expression of GFP-tagged proteins for the study of protein localization (Matsuyama *et al.*, 2006). Gene transcript profiles have been carried out to study gene expression through the vegetative cell cycle, sexual differentiation and responses to environmental stress (Rustici *et al.*, 2004; Mata *et al.*, 2002; Chen *et al.*, 2003; Mata and Bahler, 2006). These have been performed to see which genes are upregulated or downregulated in different phases of the cell cycle or under different physiological conditions.

1.1.1. Biology, evolution and life cycle of *S. pombe*

S. pombe is a fungus of the phylum Ascomycota. Characteristics of these cells include a fungal cell wall, a closed mitosis (intact nuclear membrane during mitosis) and an ascus-type sporangium (the structure in which sexual spores are enclosed; an ascus) (Sipiczki, 1995). *S. pombe* was the first of the fission yeasts to be discovered and the one on which the genus “fission yeast” was founded (Mitchison, 1957). The other two are *S. japonicus* and *S. octosporus* (Sipiczki, 1995). Interestingly, early phylogenetic analysis using rRNA internal transcribed spacer sequences, suggested that the fission yeasts were so divergent from that of *S. cerevisiae* (budding yeast) that they were initially grouped with metazoans rather than with yeasts or other fungi. Indeed, many *S. pombe* proteins turned out to be more similar to their mammalian homologues than to their *S. cerevisiae* homologues (Sipiczki, 1995). Thus, despite being an ascomycete like *S. cerevisiae*, this fission yeast is not closely related to the budding yeast. For this reason, processes such as cell cycle, chromosome structure, and RNA

splicing in *S. pombe* are more similar to that of mammalian cells than to its more heavily studied fungal counterpart, *S. cerevisiae*.

The genome of *S. pombe* is 14 megabases (Mb) in size (Moreno *et al.*, 1991, Wood *et al.*, 2002). It has 3 chromosomes of 5.7, 4.6 and 3.5 Mb. *S. pombe* cells are approximately cylindrical, typically 3-4 μm in width and 7-15 μm in length for haploid wild-type strains. Haploid cells divide when reaching approximately 12-15 μm in length. The fission yeast cell cycle is similar to that of other eukaryotes in that it has discrete S (synthesis) and M (mitotic) phases as well as the G1 and G2 gap phases. G2 is the longest phase of the cell cycle accounting for 0.7 units of a cell cycle, whereas the other phases each account for only about 0.1 units each (Moreno *et al.*, 1991). *S. pombe* has a very short G1 phase compared to other organisms. In *S. pombe*, cell separation takes place as the cell is undergoing another round of DNA synthesis (S phase) (Moreno *et al.*, 1991). Wild-type *S. pombe* isolates are homothallic (h^{90}), meaning that these strains can switch mating type between h^+ and h^- every other generation (Moreno *et al.*, 1991). The *S. pombe* strains commonly used for genetic analysis have been derived from the wild-type strains 972 h^- , 975 h^+ and 968 h^{90} , which all are isogenic (Moreno *et al.*, 1991). When grown in nutrient rich medium at 25°C, these strains have a cell duplication time of about 3 hours. Strains that are not homothallic are either of the P (h^+) or M (h^-) mating types (Moreno *et al.*, 1991). The mating type of the cell is determined by the allele present at the *mat1* locus: *mat1-P* in P cells and *mat1-M* in M cells (Arcangioli and Thon, 2004). Under poor nutrient conditions, low nitrogen, cells arrest in G1. Pheromones are then secreted by the cells prior to mating (i.e., P-factor is secreted from P cells and M-factor is secreted from M cells) (Nielson, 2004). The

pheromones bind to surface receptors on the cell of the opposite mating type. The pheromone-receptor interaction then activates an intracellular MAP kinase signal cascade, preparing cells for mating (Yamamoto, 2004). Two cells of the opposite mating type fuse, cell tip to cell tip. The nuclei from both haploid cells migrate towards one another and fuse, forming a diploid zygote (Yamamoto, 2004). A subsequent round of DNA replication is initiated raising the DNA content from 2C to 4C, where 1C represents the amount of DNA in a haploid cell in G1 (Yamamoto, 2004). Meiosis I and II occur followed by sporulation. Four haploid spores are released and will remain as spores as long as the environment remains nutrient poor. Spores will germinate given sufficient nutrients for vegetative growth. The diploid zygote can enter a vegetative cell cycle if nutrient rich conditions are maintained. From the vegetative cycle, diploids can be induced to form azygotic asci by nutrient starvation. Diploids divide at longer lengths (20-25 μm) and widths (4-5 μm) than haploid cells (Moreno *et al.*, 1991). The haploid and diploid life-cycles of *S. pombe* cells, as well as mating and sporulation, are summarized in Figure 1.1.

1.1.2. General characteristics of the *S. pombe* cell cycle

In *S. pombe*, cell division control (*cdc*) mutants have been critical in the identification of gene products required for various aspects of the cell cycle. First described in *S. cerevisiae* (Hartwell *et al.*, 1970), and later in other organisms including fission yeast (Nurse *et al.*, 1976), these mutants defined genes whose products were

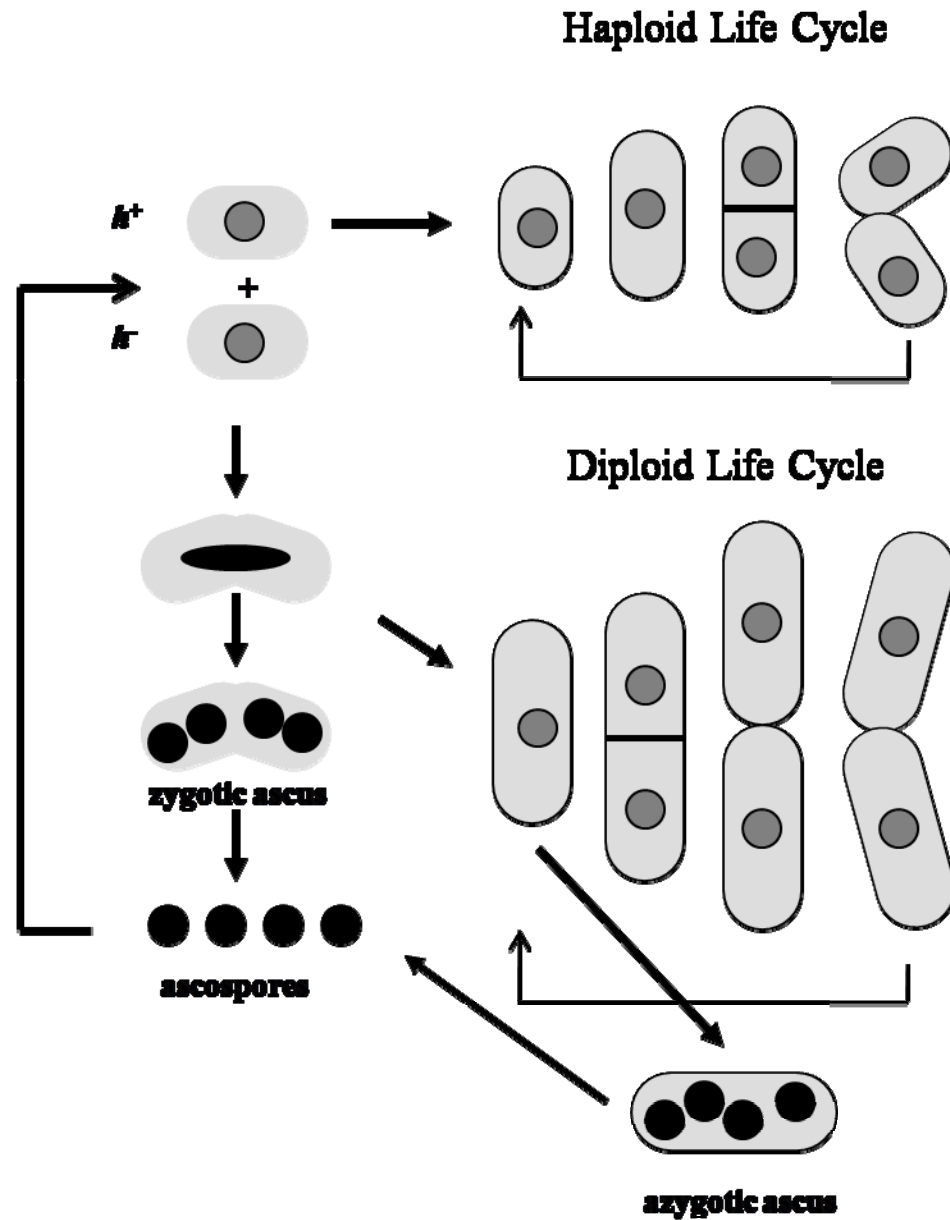


Figure 1.1: Life-cycle of *S. pombe*, including mating, meiosis and sporulation.

This figure represents the haploid and diploid mitotic cycles of *S. pombe* and includes mating, meiosis and sporulation. The laboratory strains $972h^+$ and $975h^-$ grow vegetatively as haploids under nutrient rich conditions. Under conditions of nutrient starvation, such as nitrogen deprivation, cells of the opposite mating type conjugate and their nuclei fuse. Depending on the environmental conditions, the cells can then continue to grow and divide as diploids, or sporulate. Spores germinate under nutrient rich conditions. Figure adapted from Moreno *et al.*, 1991.

essential for progression through the cell cycle. Under the restrictive temperature, cells carrying these genetic mutations arrested at the cell cycle stage at which their gene product was required. For example, *S. pombe* cells with a temperature-sensitive *cdc25* mutation arrested prior to mitosis. The *cdc25* protein product was later shown to be required for the initiation of mitosis (Nurse and Thuriaux, 1980).

Start is a genetically defined point in G1 whereby cells are committed to one round of cell division (Figure 1.2) (Sudbery *et al.*, 1980). Before Start, yeast cells can undergo alternative developmental pathways. These are meiosis and sporulation, conjugation or entry into stationary phase, depending on the extracellular conditions. Cells responding to the mating pheromone secreted by cells of the opposite mating type during the conjugation process cease to divide and become synchronized at Start. This occurs in order to synchronize the two cell cycles before cell fusion during conjugation. Start was first discovered in *S. cerevisiae* through the analysis of the gene product of *CDC28*. In *S. cerevisiae*, the *CDC28* gene product halts the cell cycle at this control point in G1. Expression of the *CDC28* product is essential for the duplication of the spindle pole body on the nuclear membrane. After Start, the cell is committed to forming a mitotic spindle and the cell enters the cell cycle (Hartwell and Unger, 1977; Reed, 1980). The *CDC28* gene product is a cyclin dependent kinase and homologues have been shown to be involved in Start in various organisms. For example, the *CDC28* homologue in *S. pombe* is *cdc2* and has been shown to be required for Start (Simanis *et al.*, 1987). The initiation of Start depends on a transcription factor complex required for the transcription of genes essential for S phase onset and progression (Simanis and Nurse, 1989).

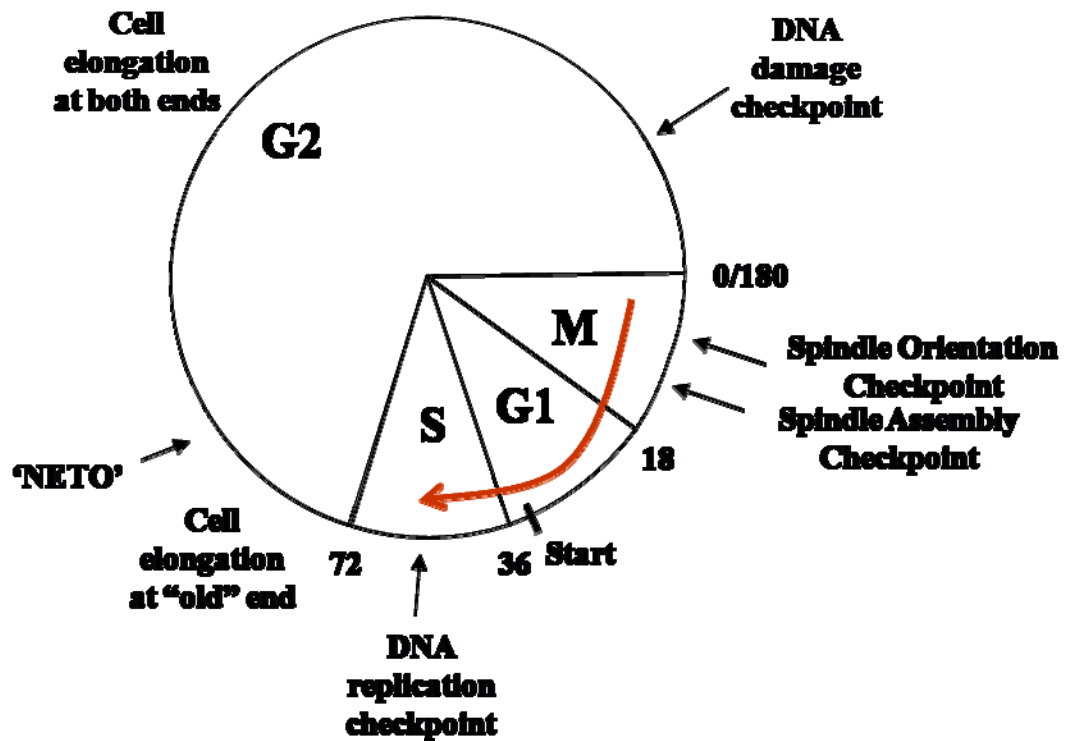


Figure 1.2: The *S. pombe* cell cycle.

The cell cycle is approximately three hours long when cells are grown in rich medium at 25°C. In *S. pombe*, cytokinesis is initiated early in mitosis and ends with cell separation (red arrow). Several cell cycle checkpoints are also indicated. During G2, the longest phase of the *S. pombe* cell cycle, cells grow initially at one end, after which growth then becomes bipolar (NETO) until mitosis. The numbers represent, in minutes, the time at which the events of the cell cycle occur. NETO: New End Take-Off.

The cell also has checkpoint mechanisms for surveillance of the progression through the cell cycle. Checkpoint mechanisms act to delay or arrest the cell cycle until a preceding step has been successfully completed. For instance, mitosis cannot begin until the DNA has been corrected for errors in sequence in response to DNA damage (Laskey *et al.*, 1989; Hennessy *et al.*, 1990). Two DNA integrity checkpoints exist in *S. pombe*: a DNA replication checkpoint and a DNA damage checkpoint. The DNA replication checkpoint ensures that DNA is replicated once in the cell cycle, and the DNA damage checkpoint ensures that the DNA is faithfully copied and repaired if damaged by environmental conditions. The DNA replication checkpoint delays cells within S phase, and the DNA damage checkpoint delays cells at the G2/M boundary (Furuya and Carr, 2003) (Figure 1.2).

After successful completion of S phase, cells enter a period of polarized cell growth called G2. In *S. pombe*, cell growth is restricted to cell ends (La Carbona *et al.*, 2006). The localization of cell growth proteins to the cortex of cell ends involves the actin cytoskeleton and depends on cell polarity pathways that are spatially and temporally regulated (La Carbona *et al.*, 2006). In early G2 phase, cells grow only at their ‘old’ end (the end where the previous division did not take place). Later in G2, cells switch to bipolar growth, termed NETO “new end take-off” (Figure 1.2; Mitchison and Nurse, 1985). NETO takes place when the cells are approximately 9-9.5 μm long (Mitchison and Nurse, 1985). Cell growth pauses, or stops, as mitosis is initiated and resumes after division (Thuriaux *et al.*, 1978).

The actin cytoskeleton is essential for cell growth, i.e., for elongation. Areas enriched with actin, such as F-actin patches, are associated with active growth zones

(La Carbona *et al.*, 2006). After cell separation, actin patches concentrate at the old end which is the growing end, as described above. At NETO, actin patches are localized to both ends and growth becomes bipolar. These are areas associated with membrane expansion and endocytosis (Gachet and Hyams, 2005). Colocalization of endocytic vesicles with actin patches is inhibited in mutants that affect actin patch integrity or by drugs that disrupt actin patches (Gachet and Hyams, 2005). Endocytosis, secretion and exocytosis are required to target enzymes for softening and remodeling of the cell wall as well as to deliver proteins and lipids required for localized cell growth (Pruyne *et al.*, 2004, for review).

Mitosis is the segregation of sister chromatids into two nuclei, a process that follows G2. In *S. pombe*, mitosis is ‘closed’ referring to the maintenance of an intact nuclear membrane. This is different from mammalian cells where the nuclear membrane breaks down at the onset of mitosis. Two checkpoints exist to monitor the progression of mitosis: the spindle assembly checkpoint (SAC) and the spindle orientation checkpoint (SOC) (Murone and Simanis, 1996; Gachet *et al.*, 2001). The SAC monitors the proper alignment of spindle microtubules with the replicated chromosomes to ensure proper segregation. The SOC monitors the actin cytoskeleton, which aids in maintaining the alignment of the spindle (Gachet *et al.*, 2001). Both checkpoints ensure that the sister chromatids are faithfully distributed to each nucleus.

Following mitosis, the binucleate cell must physically separate and distribute its cytoplasmic constituents in order to form two cells. This process is called cytokinesis. Cytokinesis occurs through a “purse-string” like separation mediated by an actin-myosin based contractile ring. Following the assembly of the contractile ring, two

membrane delimited compartments must be formed. Since *S. pombe* has a cell wall, a septum cell wall must be built for proper cell division. During contractile ring formation, actin patches disappear from the poles of the cell and actin then forms the contractile ring. Following the creation of two daughter cells, the contractile ring disassembles and actin patches reappear on each side of the septum (Marks *et al.*, 1986; Arai and Mabuchi, 2002). Initiation of contraction and septation are regulated by a signaling cascade called the septation initiation network (SIN). Cell wall hydrolyzing enzymes then digest the primary septum so that the two membrane delimited cells can physically separate. This essential phase of the cell cycle, cytokinesis, is the main focus of this research work.

1.1.3. Cytokinesis in *S. pombe*

S. pombe divides by medial fission similar to higher eukaryotes. This is in contrast to *S. cerevisiae* which divides by budding. In *S. pombe*, there are a series of events required for cytokinesis to occur as described in Figure 1.3A (Balasubramanian *et al.*, 2000; Chang, 2001). During interphase, microtubules are organized longitudinally into cytoplasmic microtubule bundles (Figure 1.3A). At the onset of mitosis, the cytoplasmic microtubules relocate and condense to form an intra-nuclear mitotic spindle (Figure 1.3A, 1). At this point, an actin-myosin contractile ring begins to form perpendicular to the long axis of the cell at the position around the predivisional nucleus (Figure 1.3A, 1). The process of contractile ring assembly occurs in ~35 minutes at 31°C (Figure 1.3B) (Wu *et al.*, 2003). Following nuclear division, more specifically anaphase in *S. pombe*, the medial actin-myosin ring begins to contract as

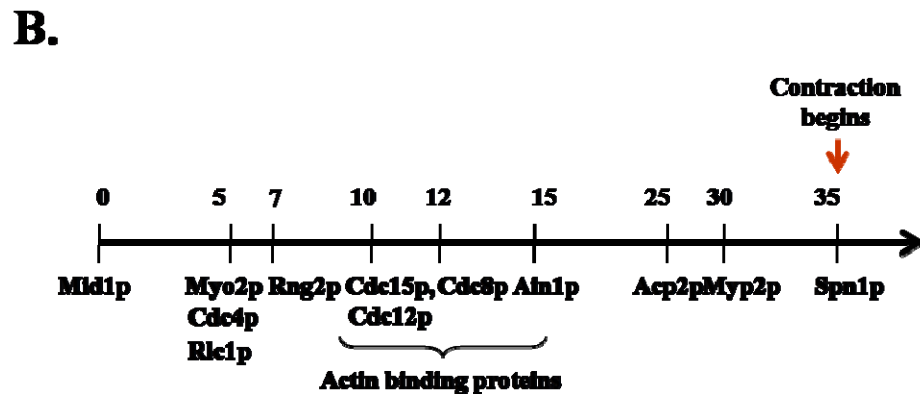
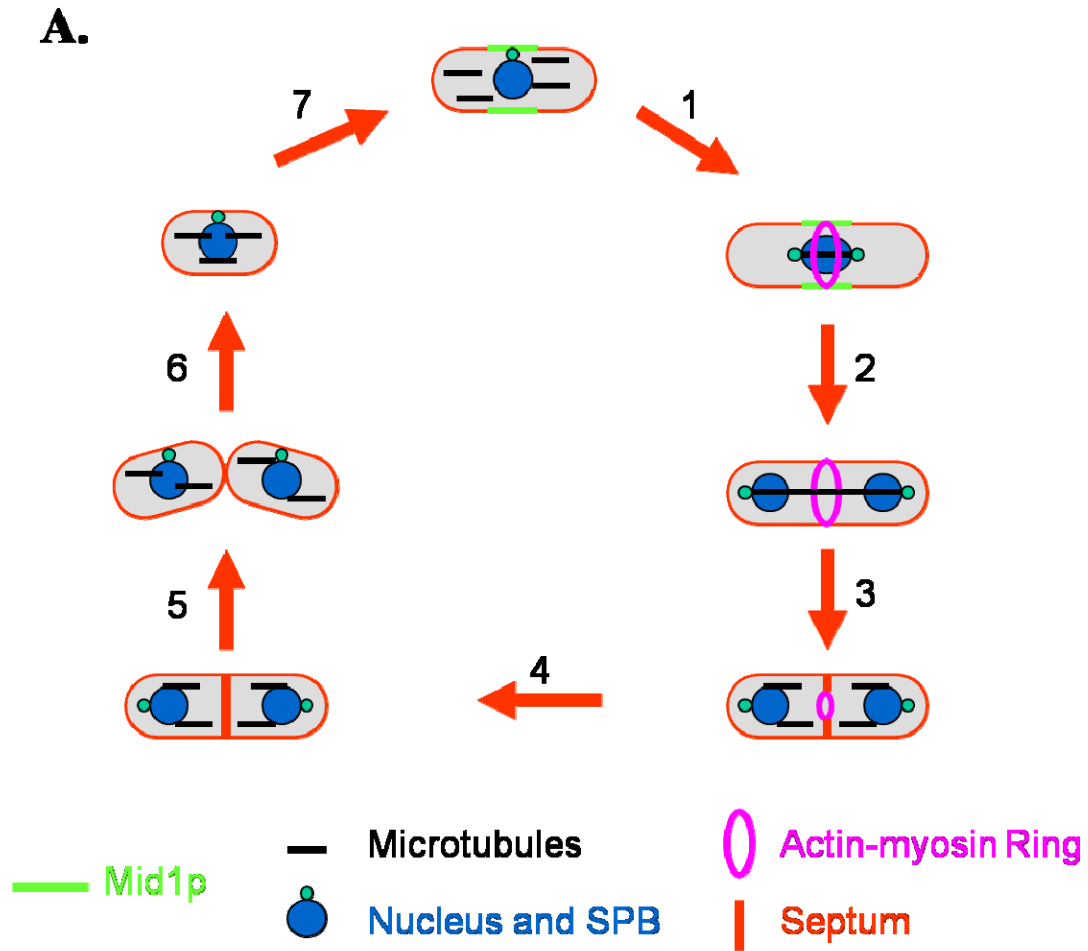


Figure 1.3: Cytokinesis in *S. pombe*.

(A) Overview of cytokinesis in fission yeast. Figure is adapted from Balasubramanian *et al.*, 2000. (B) Time line (in minutes) of the recruitment of contractile ring proteins to the medial region. Formation of the Mid1p plaques at the medial region arbitrarily marks time 0. Figure is adapted from Wu *et al.*, 2003.

new septum cell wall material is deposited in the region marked by the ring (Figure 1.3A, 3). Cell division is completed when the ring closes, the cell wall septum is completed (Figure 1.3A, 4), and the primary septum is hydrolyzed (Figure 1.3A, 5), generating two daughter cells (Figure 1.3A, 6).

1.1.3.1. Determination of the cell division site

In *S. pombe*, the nucleus provides the main positional information for the formation of the contractile ring, unlike in animal cells where the positional information of the ring is dictated by the spindle microtubules. This is likely due to the closed mitosis of fission yeast, where the spindle is intranuclear and does not have the opportunity to communicate with the cell surface, unlike in animal cells where the mitotic spindle is exposed due to the breakdown of the nuclear membrane (Chang, 2001). In *S. pombe*, microtubules position the nucleus in the middle of the cell (Tolic-Norrelykke *et al.*, 2005; Daga and Chang, 2005). The evidence that the nucleus provides the positional information for the formation of the ring comes from studies where displacement of the nucleus results in displaced rings. Moving the nucleus to one side of the cell using optical tweezers, or centrifugation, showed that movement of the nucleus off-center also moved the position of the cell division plane off-center (Tolic-Norrelykke *et al.*, 2005; Daga and Chang, 2005). Interestingly, the displaced nucleus displaced the division site only in cells that were pre-mitotic (Tolic-Norrelykke *et al.*, 2005). For example, prometaphase nuclei that were displaced did not alter the position of the division site. Thus, the positional information of the contractile ring is dictated before mitosis begins.

Two proteins, Mid1p and Plo1p, play major roles in determining the site of cell division in *S. pombe*. Mutations in either of these proteins causes the cells to divide asymmetrically, with ring formation frequently initiated near the cell poles (Bahler *et al.*, 1998a). Mid1p, also known as Dmf1p (Sohrmann *et al.*, 1996; Paoletti and Chang, 2000), is similar in sequence to mammalian and insect anillin which appears to perform similar functions. The term anillin stems from the Spanish word for “anillo”, which means ring (Field and Alberts, 1995). *Drosophila* anillin is located in the nucleus in interphase cells, like Mid1p, except in the syncytial embryo where it is always cytoplasmic. During metaphase, anillin is present in the cytoplasm and cortex and during the anaphase-telophase transition, it is highly enriched at the cleavage furrow along with myosin II. Field and Alberts (1995) did not report an association between myosin II and anillin. Anillin in insect cells binds actin through amino acids 127-371 and has been shown to bundle actin filaments. Thus, a role of anillin may be to stabilize actin filaments during contraction (Field and Alberts, 1995). Overall, insect anillin appears to function similarly to Mid1p except that it has not been shown to be involved in division site placement.

Mid1p is non-essential, unlike Plo1p which is an essential gene. Mid1p has a basic region that contains several nuclear localization signals (NLS) (amino acids 681-710) and 2 nuclear export signals (NES) (Sohrmann *et al.*, 1996; Paoletti and Chang, 2000). Also, it has a C-terminal pleckstrin homology domain (PH) (amino acids 805-896) (Sohrmann *et al.*, 1996; Paoletti and Chang, 2000). PH domains are often observed in signaling or cytoskeletal proteins and are involved in protein-protein, or protein-lipid interactions. In wild-type cells, septa form perpendicularly to the

longitudinal axis of the cell. The temperature-sensitive mutant *mid1-366* was examined for its phenotype at the restrictive temperature. The nuclei of these cells remained centrally located, however, the position and angles of the septa were abnormal (Sohrmann *et al.*, 1996). Furthermore, staining with rhodamine-conjugated phalloidin for actin ring structures, and anti-Cdc15p antisera for contractile ring structures, indicated that abnormally placed rings were associated with the septa. Cdc15p is a component of the contractile ring in *S. pombe*. Cells carrying *mid1-366* synchronized for late G2 at 25°C by elutriation, and shifted to 36°C, initiated septation in the medial region of the cells again showing that the position of the ring, and therefore septum, is dictated before mitosis begins (Sohrmann *et al.*, 1996).

Mid1p is nuclear in interphase cells, and is shuttled outside of the nucleus to a cortical band above the predivisional nucleus early in mitosis before the contractile ring forms. Mid1p then coalesces into a ring at anaphase A (Sohrmann *et al.*, 1996; Wu *et al.*, 2003). Once the contractile ring has begun contraction, and the cell is close to completing septation, Mid1p once again becomes nuclear (Sohrmann *et al.*, 1996; Wu *et al.*, 2003). When Mid1p localization changes from nuclear to medially localized at the cell cortex, it becomes hyperphosphorylated (Sohrmann *et al.*, 1996). This hyperphosphorylation occurs prior to contractile ring formation. At septation, when Mid1p again becomes nuclear, Mid1p reverts to its hypophosphorylated form. Mid1p cortical plaque formation does not require the presence of the F-actin ring, microtubules or Cdc15p function. However, mutations in Mid1p are synthetically lethal with certain contractile ring components (*cdc3*, *cdc4* and *cdc8*) suggesting that Mid1p may act to tether the ring to the plasma membrane (Sohrmann *et al.*, 1996).

As mentioned previously, Mid1p has 2 NES, and when both were mutated, Mid1p localization to the cortex was diminished (Paoletti and Chang, 2000). Furthermore, this diminished localization to the cortex resulted in septation defects. Thus, Mid1p localization to the cortex is required for its function in division site placement. A mutation in the Mid1p NLS diminished Mid1p localization to the nucleus, and produced no effect on division site placement in these cells (Paoletti and Chang, 2000). Finally, the PH domain of Mid1p, which is located at the C-terminal end of the protein, was deleted and it was shown that it was not required for the recruitment of Mid1p to the nucleus or to the plasma membrane and therefore not required for its function (Paoletti and Chang, 2000). Thus, Mid1p is a protein involved in specifying the division site in *S. pombe* by shuttling out of the nucleus before mitosis begins to a cortical plaque at the medial region of the cell. The function of the PH domain is unknown, but not essential for Mid1p function.

Plo1p is also required for proper placement of the division site in *S. pombe* cells. Plo1p is a Ser/Thr protein kinase homologous to *Drosophila* polo kinases, where they were first identified (Bahler *et al.*, 1998a). Three *plo1* temperature-sensitive mutants were identified that displayed defects in ring and septum placement, with *plo1-1* being the most severe. In *plo1-1* cells at the non-permissive temperature, Mid1p failed to form a broad cortical band above the predivisional nucleus (Bahler *et al.*, 1998a). Also, when *plo1* was ectopically expressed, the majority of the cells showed Mid1p localization to a cortical band above the predivisional nucleus (Bahler *et al.*, 1998a). Furthermore, Mid1p displayed retarded mobility in electrophoretic assays when *plo1* was overexpressed. This suggests that Mid1p exit from the nucleus early in

mitosis is dependent on activation by Plo1p. Mid1p was shown to interact with the C-terminal domain of Plo1p in yeast two-hybrid assays (Bahler *et al.*, 1998a). Thus, Mid1p may be a substrate for Plo1p protein kinase activity.

Another protein required for the proper placement of the ring and septum is Pom1p. Pom1p is a DYRK (*dual specificity tyrosine phosphorylation regulated kinase*)-family protein kinase (Padte *et al.*, 2006). Pom1p loss-of-function temperature-sensitive mutants grow only in a monopolar manner and display defects in ring and septum positioning (Bahler and Pringle, 1998; Bahler and Nurse, 2001). Pom1p localizes to the plasma membrane and to cell ends (enriched at the non-growing end) during interphase (Bahler and Pringle, 1998; Bahler and Nurse, 2001). During cytokinesis, Pom1p localizes to the septum. Genetic analysis of double-mutants of Plo1p, Mid1p and Pom1p suggest that Mid1p may function in a common pathway with Plo1p, whereas Pom1p may have a distinct and complementary role in septation (Bahler *et al.*, 1998). In *pom1* mutants, Mid1p is distributed on over half of the cell cortex including the non-growing end (Padte *et al.*, 2006). This abnormal distribution of Mid1p in interphase cells leads to the formation of misplaced rings and septa (Padte *et al.*, 2006). This suggests that both cues from the nucleus and cell tips act to specify the position of the division plane.

Finally, a genetic screen identified 16 mutants defective in ring placement (Edamatsu and Toyoshima, 1996). It is unclear whether the *plo1*, *mid1* or *pom1* gene products were isolated in this screen as the mutated genes were not identified. As yet, there is no further information on the contribution of these genes to division site placement.

1.1.3.2. Components of the contractile ring

Following the formation of the Mid1p cortical plaque above the predivisional nucleus, protein components of the contractile ring are recruited to the medial region, through the activation by Plo1p. Many proteins are required for the assembly of the contractile ring and the sequential events of contractile ring assembly were determined for 12 proteins involved in cytokinesis (Wu *et al.*, 2003) (Figure 1.3B). As previously stated, Mid1p is the first protein to appear at the medial region before mitosis begins. Following Mid1p localization to the medial region as a cortical plaque above the predivisional nucleus, the conventional type II myosin Myo2p, and its light chains, Cdc4p and Rlc1p, appear at the medial region. The human IQGAP1 homologue Rng2p follows Myo2p localization by approximately 2 minutes. Approximately 8 minutes later, the PCH protein, Cdc15p, and formin, Cdc12p, localize to the medial region, followed by the tropomyosin, Cdc8p, and α -actinin, Ain1p. These proteins are actin associated proteins and thus mark the recruitment of actin to the medial region. In this study, GFP-tagged actin did not localize to the medial region and was consequently monitored indirectly through the actin associated proteins Cdc15p, Cdc12p, Cdc8p and Ain1p. Approximately 12 minutes later, the capping protein Acp2p joins the ring, followed by the unconventional myosin, Myp2p, and the septin, Spn1p. In addition to these proteins, other proteins contribute to cytokinesis such as the fimbrin, Fim1p, the PCH protein, Imp2p, profilin, Cdc3p, the Arp2/3 complex, Mid2p, and the Rho GTPases Rho3p and Rho4p.

1.1.3.3. Assembly of the contractile ring

The time required for the recruitment of contractile ring proteins after Mid1p cortical plaque formation is 3-3.5 minutes (Figure 1.3B) (Wu *et al.*, 2003). Several proteins required for actin-myosin ring assembly have been identified in *S. pombe*. *S. pombe* strains carrying mutations in genes whose protein products are required for contractile ring assembly demonstrated misoriented and aberrant looking contractile rings. One key structural and motor protein required for contractile ring assembly is the type II myosin, Myo2p (Naqvi *et al.*, 1999; Motegi *et al.*; 2000; Wu *et al.*, 2003). In cells, Myo2p exists as a hexameric complex. The hexamer is composed of two Myo2p heavy chains and four light chains, of which two are essential light chains (Cdc4p) and two are regulatory light chains (Rlc1p). The four light chains bind to the flexible neck region of myosin (Naqvi *et al.*, 1999). Research on mechanical force transduction performed in other organisms in muscle cells suggests that myosin essential light chains play a structural role in facilitating myosin-actin sliding filament movement (Trybus, 1994). This is in contrast to the regulatory light chain, which acts to regulate or modulate the myosin heavy chain (Trybus, 1994; Sherwood *et al.*, 2004; Zhi *et al.*, 2005; Ryder *et al.*, 2007).

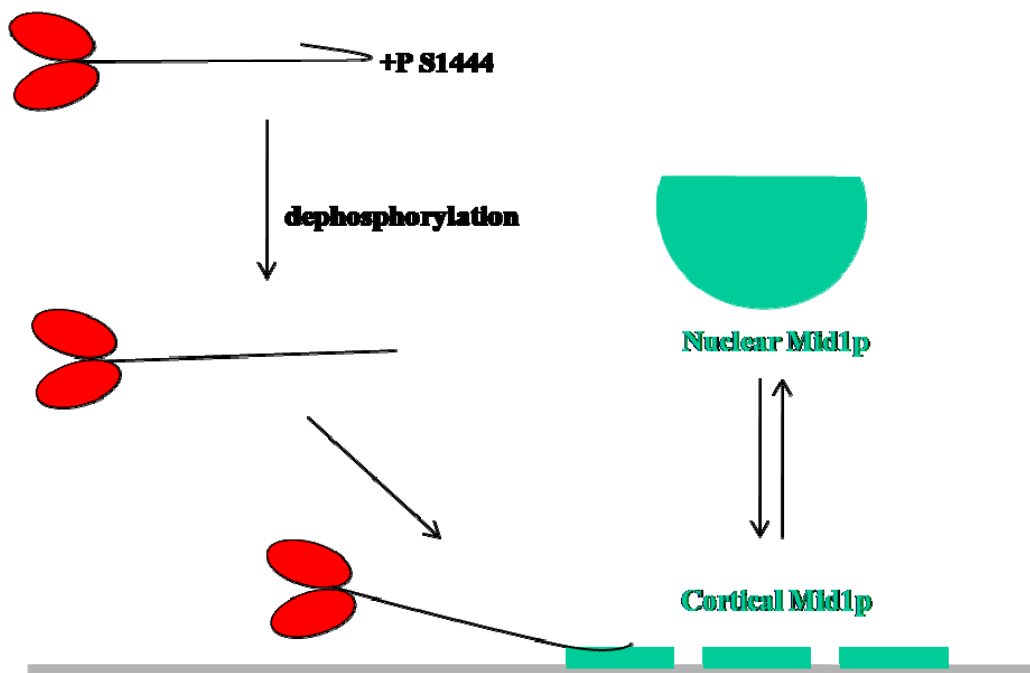
The N-terminal region of myosin is composed of a globular ATPase domain which binds actin. The formation of a coiled-coil involving the C-terminal region of myosin polypeptides stabilizes the myosin heavy chain dimer. The C-terminal domain of Myo2p is essential for its localization to the contractile ring (Mulvihill *et al.*, 2001; Motegi *et al.*, 2004). Phosphorylation and dephosphorylation events at the tail end of myosin are required for its recruitment to the medial region of the cell. The phosphorylation of serine 1518 in the myosin tail region is required for assembly of

Myo2p into rings at the medial region of the cell (Mulvihill *et al.*, 2001). Myosin also has another phosphorylation site, serine 1444 which is dephosphorylated early in mitosis (Motegi *et al.*, 2004). A Myo2p serine 1444 to alanine mutant, which mimics the dephosphorylated form of myosin that is normally present during mitosis, causes myosin to accumulate at the division site prematurely (during G2 and mitosis) followed by the recruitment of actin and the formation of an acto-myosin ring. Mid1p was also observed to interact with Myo2p S1444A and not the Myo2p S1444D mutant which mimics the phosphorylated S1444 form of myosin. It is thought that the dephosphorylation of myosin on serine 1444 relieves the intramolecular inhibition of myosin liberating the C-terminus for assembly into a ring at the medial region of the cell (Figure 1.4) (Motegi *et al.*, 2004). In *D. discoideum*, non-muscle myosin has a bent hinge-like region in its C-terminal domain, like *S. pombe* Myo2p (Motegi *et al.*, 2004). Three phosphorylation events at the C-terminal region promote bending of the tail region inhibiting the assembly of myosin into filaments. A similar model has been proposed for *S. pombe* Myo2p. During interphase, Myo2p is phosphorylated at serine 1444, suppressing the interaction with Mid1p due to its bent conformation. Once mitosis begins, dephosphorylation of Myo2p S1444 exposes the C-terminal region allowing the interaction with Mid1p to occur (Figure 1.4A). Myo2p then interacts with actin filaments coalescing the ring into a tight band through actomyosin motor activity (Figure 1.4B). This is consistent with evidence showing that Myo2p mutants defective for ATPase activity fail to assemble a condensed contractile ring and mutants of *myo2* defective in binding actin filaments still accumulate at the division site (Naqvi *et al.*, 1999).

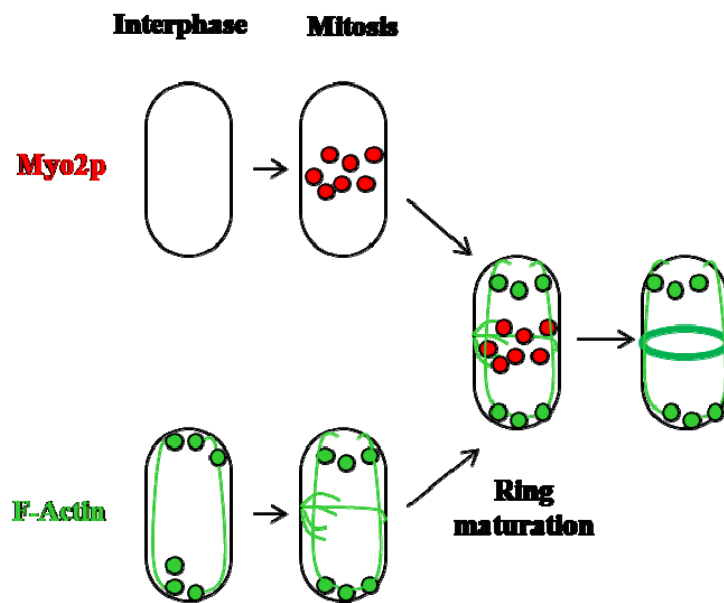
Figure 1.4: Assembly of Myo2p at the contractile ring in *S. pombe*.

(A) Mid1p shuttles out of the predivisional nucleus to the cell cortex above the predivisional nucleus. During interphase, phosphorylation of Myo2p on S1444 inhibits Myo2p from interacting with Mid1p. This is because phosphorylation of Myo2p at S1444 causes the Mid1p binding site to be concealed through the bending of the Myo2p tail. At the beginning of mitosis, dephosphorylation exposes the C-terminal region of Myo2p allowing the tail region to interact with Mid1p at the cell cortex overlying the nucleus. (B) Myo2p accumulates as dots at the medial region early in mitosis. This localization is dependent on Mid1p, but independent of F-actin. F-actin forms as an aster/spot-like structure at the medial cortex independent of Myo2p. Myo2p dots recruit and/or stabilize F-actin at the medial cortex. The cortical networks of F-actin and Myo2p are compacted into a tight ring by actomyosin motor activity. Figure adapted from Motegi *et al.*, 2004.

A.



B.



Following Myo2p localization to the actomyosin ring, Rng2p becomes recruited to the contractile ring. Rng2p is a protein related to the human IQGAP protein. Human IQGAP proteins are effectors of the Rho family of GTPases (Eng *et al.*, 1998). IQGAP proteins will be discussed in greater detail in section 1.1.3.6.2. Rng2p recruitment to the medial region is followed by the recruitment of actin and actin associated proteins such as Cdc15p (PCH), Cdc12p (formin), Cdc8p (tropomyosin), Ain1p (α -actinin) and Acp2p (actin capping protein) (Wu *et al.*, 2003). Thus, actin recruitment and polymerization at the ring occurs after myosin has localized to the medial region (Wu *et al.*, 2003).

There are two main hypotheses regarding the formation of the contractile ring. The first hypothesis states that the anillin-like protein Mid1p recruits a progenitor spot carrying myosin II (Myo2p), formins (Cdc12p) and PCH (Cdc15p) proteins to the division site, followed by the extension of a cable from the progenitor spot which forms around the circumference of the cell forming the contractile ring (Wu *et al.*, 2006). The second hypothesis states that the contractile ring components form a broad band of nodes around the middle of the cell which then coalesces into a contractile ring (Wu *et al.*, 2006). These nodes are composed of 7 proteins: Mid1p, Myo2p with Cdc4p and Rlc1p, Rng2p, Cdc15p and Cdc12p. There is strong evidence for the latter hypothesis (Vavylonis *et al.*, 2008). Fluorescence microscopy with functional fusion proteins expressed at normal levels showed that in G2/M cells, most of these proteins formed a broad band of nodes around the equator of the cell which then coalesced into a ring. Mid1p was shown to be required for the formation of the Myo2p, Rng2p, Cdc15p and Cdc12p nodes (Wu *et al.*, 2006; Vavylonis *et al.*, 2008).

Before mitosis begins, Mid1p initiates the assembly of ~75 nodes around the medial region of the cell, first recruiting Myo2p with its light chains, as well as Rng2p (Wu *et al.*, 2003; Wu *et al.*, 2006). The role of Rng2p is unclear in this process. The Cdc15p and Cdc12p proteins are then recruited to the medial region of the cells. Cdc12p is a formin that forms homodimers that nucleate unbranched actin filaments remaining attached to their barbed ends (Kovar *et al.*, 2005). Each node has been observed by microscopy and flow cytometry along with quantitative immunoblots, to contain ~20 Mid1p proteins, 20 dimers of myosin II with light chains, 20 IQGAP Rng2p proteins, 20 PCH Cdc15p proteins, 2 dimers of Cdc12p and ~250 molecules of Cdc4p proteins in molar excess of the Myo2p binding sites (Wu *et al.*, 2006). Within these nodes, orderly interactions between the proteins are thought to occur. For example, Myo2p interacts with actin filaments and Mid1p, and Myo2p tails bind to each other forming a Myo2p homodimer (Naqvi *et al.*, 1999; Motegi *et al.*, 2004). Cdc4p interacts with Myo2p and Rng2p (Naqvi *et al.*, 1999; D'Souza *et al.*, 2001) and the N-terminal regions of Cdc15p and Cdc12p interact with each other forming a heterodimer (Chang *et al.*, 1997; Carnahan and Gould, 2003). Cdc12p also forms a homodimer with itself. Cdc4p is required to recruit both Myo2p and Rng2p to the nodes (Wu *et al.*, 2006).

Actin is required for the nodes to coalesce into a tight ring at anaphase A (Wu *et al.*, 2003; Wu *et al.*, 2006). The localization of Cdc12p is proposed to play a central role in this process. For example, formins nucleate actin filaments, remaining bound to the barbed ends presumably anchoring the actin filaments to the nodes (Kovar *et al.*, 2005). The presence of other actin polymerization proteins at this site has not been

addressed as of yet (i.e., WASP or SCAR/WAVE proteins). The interaction of Myo2p with actin is then thought to pull together the nodes into a tight contractile ring (Figure 1.4B) (Wu *et al.*, 2006). This is consistent with the observation that temperature-sensitive mutant versions of the actin binding proteins Cdc12p (formin) and Cdc3p (profilin) fail to form compact actin rings during cytokinesis at the non-permissive temperature (Wu *et al.*, 2006). The localization of Cdc3p in these nodes was not looked at in this study, but Cdc12p interacts with Cdc3p and Cdc15p (Chang *et al.*, 1997; Carnahan and Gould, 2003). The Cdc12p, Cdc15p and Cdc3p interactions at the medial region are thought to be important for actin polymerization (Wu *et al.*, 2003; Wu *et al.*, 2006). The compaction of the actin ring lasts ~10 minutes (Wu *et al.*, 2003).

Actin is not required to maintain the integrity of the ring once it has formed since the addition of actin depolymerizing agents does not destabilize contractile rings containing Myo2p and its associated light chains Cdc4p and Rlc1p (Naqvi *et al.*, 1999). This suggests that other proteins maintain the integrity of the ring and its association with the plasma membrane. However, Myo2p localization is dependent on Mid1p, a cortically associated protein. Myo2p has been shown to interact with Mid1p in cell lysates suggesting that the Myo2p ring may be anchored to the cell membrane by Mid1p (Motegi *et al.*, 2004). This provides a mechanism as to how the contractile ring is linked to the cell membrane. During anaphase B, the contractile ring matures through the addition of the non-conventional myosin II, Myp2p, and the septin, Spn1p (Wu *et al.*, 2003). Myp2p is unconventional in that it is monomeric with a proline rich hinge region that allows the tail to fold back on itself forming a rod-shaped, anti-parallel, coiled-coil (Bezanilla and Pollard, 2000).

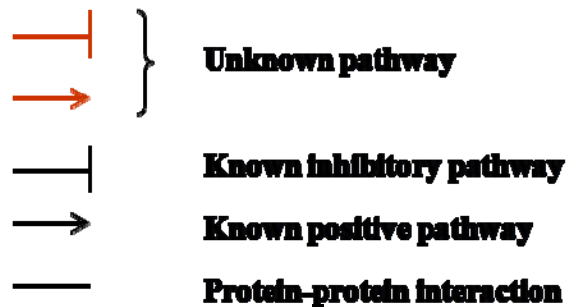
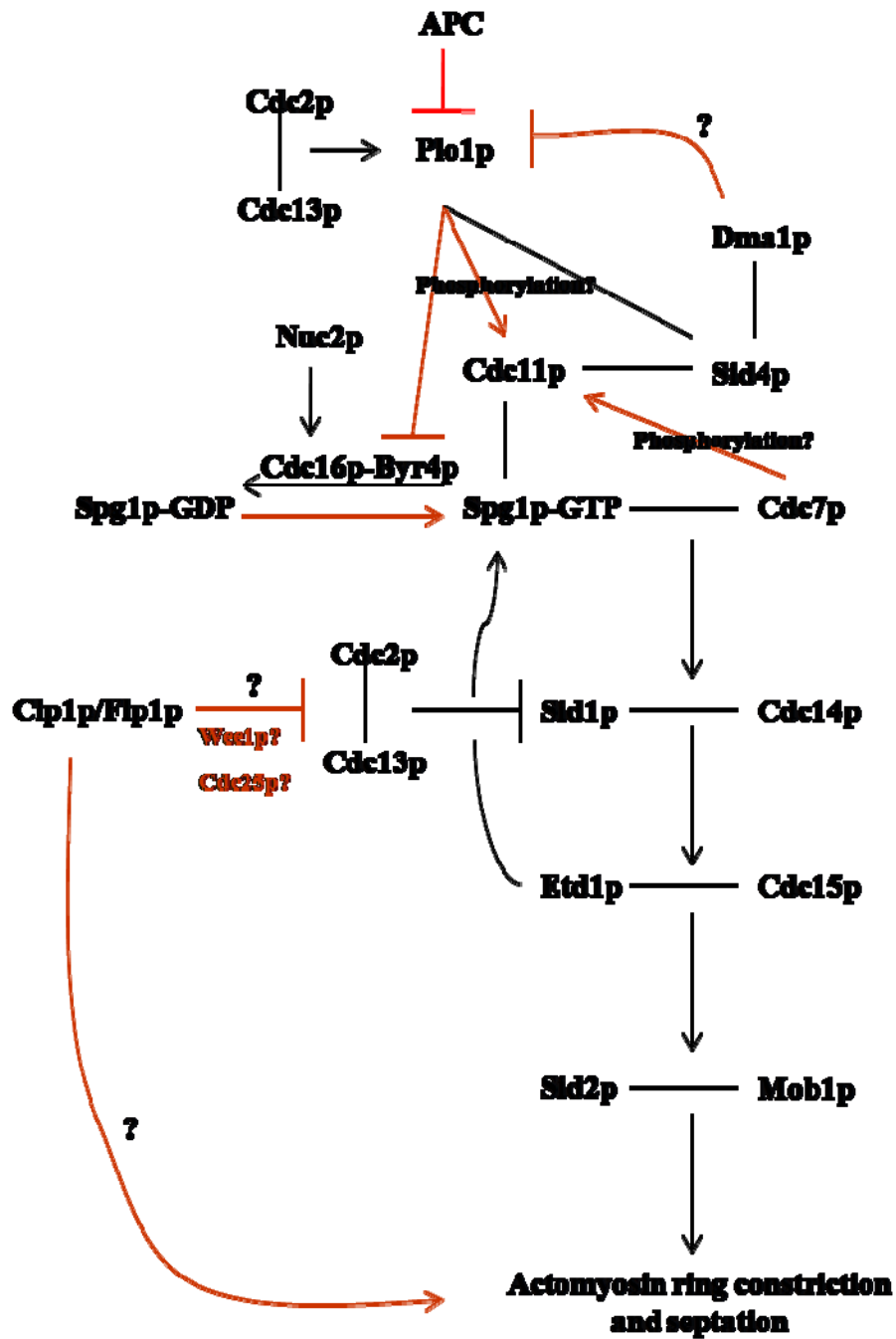
It is an issue of debate whether the contractile ring actually generates the force required to divide the binucleate cell in half (Chang, 2000). The medial ring does shrink during cytokinesis (McCollum *et al.*, 1995). However, mutants of the ATPase domain of myosin are defective in ring assembly and so it is not certain whether this region is required for contraction (Naqvi *et al.*, 1999). A temperature-sensitive allele in which myosin ATPase activity is conditionally absent would be beneficial in elucidating the role of myosin ATPase activity during cytokinesis, but has not been obtained thus far. In mammalian cells, inhibition of myosin II heavy chain activity through the use of blebbistatin, a myosin II inhibitor, inhibits contraction of the contractile ring providing evidence that the ring closes due to myosin motor activity (Straight *et al.*, 2003). This however, has not been shown in *S. pombe*. Also, closure of the ring is coupled to septation, and thus it is plausible that the septum may contribute to some of the forces involved in cytokinesis, and the primary role of the ring may be to mark the site of septation, and to coordinate the process of bringing together plasma membranes (Chang, 2000).

1.1.3.4. Regulation of contraction and septation in *S. pombe*

Contraction and septation in *S. pombe* are regulated by a signaling cascade emanating from the spindle pole bodies (SPB) composed of a GTPase, a two component GAP (GTPase activating protein) and three protein kinases (Bardin and Amon, 2001). This signaling cascade is known as the septation initiation network (SIN) (Figure 1.5). Nine mutant strains have been identified in *S. pombe* that are septation initiation

Figure 1.5: The septation initiation network (SIN) in *S. pombe*.

This figure summarizes the SIN in *S. pombe* which regulates the initiation of contraction and septation. Spg1p is a molecular switch, where at anaphase, signaling is induced by the anaphase promoting complex (APC). Activation of Spg1p leads to a signaling cascade which results in Sid2p-Mob1p localizing to the medial region. Sid2p, a protein kinase which acts late in the signaling cascade, phosphorylates currently unknown substrates. Signaling is stopped partly due to the ubiquitination and degradation of Etd1p.



deficient (*sid*), forming highly elongated, multinucleate (4-6 nuclei) cells: *sid1*, *sid2*, *sid4*, *cdc7*, *cdc11*, *cdc14*, *mob1*, *plp1* and *spg1* (Figure 1.5) (Bardin and Amon, 2001). At the restrictive condition, these mutant strains form a non-contractile actomyosin ring, and no septum. There are also two negative regulators of this pathway. The *byr4* and *cdc16* mutant strains produce multiseptated cells (Bardin and Amon, 2001). A homologous pathway exists in *S. cerevisiae* known as the mitotic exit network (MEN) (Bardin and Amon, 2001; Dumitrescu and Saunders, 2002). However, the genes involved in the MEN regulate multiple events occurring during mitotic exit, whereas the genes involved in the SIN function to strictly control actomyosin ring contraction and septation (Bardin and Amon, 2001).

The SIN pathway comprises the Sid4p-Cdc11p complex. It has been suggested that the Sid4p-Cdc11p complex may act as a scaffold localizing other SIN proteins to the SPB. Sid4p is a protein of predicted molecular weight of 76.4 kDa that has no sequence similarities to other known proteins in the NCBI database (Chang and Gould, 2000). Sid4p was found to localize to the SPBs independent of microtubules and other SIN proteins. In contrast, the localization of all other SIN proteins required Sid4p for their recruitment to the SPB. Analysis of the protein sequence demonstrated that there was a putative presence of two coiled-coil domains within the C-terminal half of Sid4p, suggesting dimerization, which was further demonstrated by *in vitro* translation analyses (Chang and Gould, 2000). Coiled-coil domains are common amongst SPB proteins (Chang and Gould, 2000). Thus, it is postulated that Sid4p acts as a scaffold protein on which other SIN proteins may bind. Like *sid4*, *cdc11* is an essential gene. Cdc11p is also required for the localization of all other SIN proteins

(except Sid4p) to the SPB (Chang and Gould, 2000). Sid4p interacts with Plo1p, and Cdc11p interacts with a variety of proteins through its N-terminal region such as Spg1p, Sid2p, Cdc16p and the Cdc2p-Cdc13p complex (Morrell *et al.*, 2004).

Plo1p is an upstream activator of the SIN pathway since *plo1* mutants display septation defects similar to those of mutants of the SIN pathway (Tanaka *et al.*, 2001). Plo1p acts upstream of the Spg1p pathway and is downregulated upon mitotic exit (Tanaka *et al.*, 2001). Plo1p is recruited to the SPB, mitotic spindle and medial ring upon entry into mitosis and the spindle and medial ring localizations are lost at anaphase (Tanaka *et al.*, 2001). The Plo1p association with the SPB is strongest at the onset of mitosis, and diminishes in anaphase, disappearing when the mitotic spindle breaks down. The Plo1p association with the SPB is dependent on the interaction between the cyclin dependent kinase, Cdc2p, and cyclin, Cdc13p. The dissociation of Plo1p from the SPB is dependent on the anaphase promoting complex (APC) (Mulvihill *et al.*, 1999).

Spg1p is a Ras superfamily GTPase which is negatively regulated by the two-component GTPase activating protein (GAP), Cdc16p and Byr4p, and which signals through Cdc7p. GAPs catalyze the hydrolysis of GTP to GDP of GTPases, thereby inactivating them. Spg1p is essential for *S. pombe* viability and is found on the SPBs throughout the cell cycle (Schmidt *et al.*, 1997). Spg1p lacks the CAAX isoprenylation motif suggesting that, unlike other Ras superfamily GTPases, Spg1p is not targeted to plasma membranes by lipid anchoring (Schmidt *et al.*, 1997). Overexpression of *spg1* drives septation in G2, S phase and pre-start (G1) arrested cells suggesting that the temporal and spatial regulation of Spg1p is required for septation. In interphase cells,

Spg1p is found in its GDP bound inactive form, but upon entry into mitosis, it converts to the GTP-bound active form. Sgp1p is then active on both SPBs until anaphase B, where it converts back to its GDP-bound form at one of the two SPBs. The protein kinase Cdc7p was isolated as an interactor of Spg1p by co-immunoprecipitation and yeast two-hybrid assays (Schmidt *et al.*, 1997) and overexpression of *cdc7* has been observed to rescue *spg1* null mutants indicating that Cdc7p functions downstream of Spg1p (Schmidt *et al.*, 1997). Cdc7p is a protein kinase that is active throughout the cell cycle. The two-component GAP, Cdc16p-Byr4p, regulates the active form of Spg1p (Spg1p-GTP) which binds to Cdc7p allowing Cdc7p to signal to its downstream targets (Li *et al.*, 2000). Thus, Cdc7p activity in the SIN is regulated by its binding to Spg1p-GTP.

Both Spg1p and Cdc7p localize to both SPBs at the onset of mitosis, but at anaphase both proteins are then found on only one SPB (Schmidt *et al.*, 1997, Sohrmann *et al.*, 1998). It is the assymetrical localization of the Spg1p negative regulators Cdc16p and Byr4p which cause Spg1p to remain active on one SPB (Cerutti and Simanis, 1999). The two component GAP interacts with Spg1p (Schmidt *et al.*, 1997). Both Byr4p and Cdc16p are localized to both SPBs in interphase cells until anaphase where they localize to one SPB (Cerutti and Simanis, 1999). It still remains to be determined if Cdc16p is the catalytic subunit, or if Byr4p acts as a scaffold holding Cdc16p to Spg1p to facilitate GTP hydrolysis.

Sid1p and Cdc14p form a complex which acts downstream of Spg1p and Cdc7p, and upstream from Sid2p and Mob1p (Guertin *et al.*, 2000). Sid1p is a member of the p21 activated kinase (PAK) family of protein kinases (Guertin *et al.*, 2000).

Localization of Sid1p using GFP tagged versions showed that Sid1p distribution in interphase cells is cytoplasmic and diffuse. Sid1p also localizes to both SPBs at anaphase A and later to one SPB at anaphase B. Sid1p localization to one SPB is observed before septation initiation and prior to cell division, but only after septation is complete does the Sid1p signal disappear from the SPB (Guertin *et al.*, 2000). Furthermore, Sid1p localization is dependent on the upstream effectors: *cdc7*, *cdc11*, *cdc14*, *spg1* and *sid4*. The localization of Cdc14p was similar to that observed with Sid1p (Guertin *et al.*, 2000). It is hypothesized that one of the functions of Cdc14p is to offer a binding site on the SPB, or proteins on the SPB, to localize Sid1p, since Cdc14p possesses no known catalytic activity (Guertin *et al.*, 2000). Sid1p protein kinase activity peaked at the anaphase-telophase transition corresponding to its localization to the SPB (Guertin *et al.*, 2000). Localization of Sid1p to the SPBs is dependent on the APC and loss of Cdc2p activity, indicating that Sid1p does not localize to the SPB until Cdc13p has been ubiquitinated and degraded (Guertin *et al.*, 2000). This is unlike Cdc7p which localizes to the SPB without the requirement of Cdc13p degradation (Sorhmann *et al.*, 1998). The SIN in *S. pombe* is allowed to proceed by cyclin proteolysis, but not vice versa, by the observation that cells with mutants in the SIN undergo multiple rounds of nuclear division. Consequently, the SIN is not essential for cyclin degradation and mitotic exit. A substrate for Sid1p protein kinase activity is still unknown. However, Sid2p functions downstream from the Sid1p-Cdc14p complex and thus, Sid2p (and presumably others) could possibly be a target for Sid1p (Guertin *et al.*, 2000).

The Sid2p-Mob1p complex acts downstream of the Sid1p-Cdc14p and Cdc7p-Spg1p complexes. The Sid2p protein is also a protein kinase and its activity requires the activity of *cdc7*, *cdc11*, *cdc14*, *sid1*, *spg1* and *sid4* indicating that it acts late in the SIN cascade (Sparks *et al.*, 1999, Salimova *et al.*, 2000). Mob1p, like its interactor Sid2p, localizes to both SPBs early in mitosis (Salimova *et al.*, 2000). Localization studies of Mob1p and Sid2p in *mob1* and *sid2* mutants indicate that Sid2p is maintained on the SPB throughout the cell cycle, whereas Mob1p may be recruited independently of Sid2p (Salimova *et al.*, 2000). Immunoelectron microscopy demonstrated that Sid2p localizes to the outer cytoplasmic face of the SPB indicating that the Sid2p protein may actually be a component of the SPB (Sparks *et al.*, 1999). This localization also allows Sid2p to easily move along microtubules to the site of cell division. At the end of anaphase, Sid2p can be found not only at the SPBs, but also at the medial region as well, where Sid2p could be observed at the ring and later spanning both sides of the primary septum (Sparks *et al.*, 1999). Sid2p protein kinase activity peaks during actomyosin ring contraction and septation at the end of anaphase indicating that its protein kinase activity may regulate contraction and septation in *S. pombe* (Sparks *et al.*, 1999). Sid2p and Mob1p are exceptions to the other SIN proteins in that they localize to both SPBs. Furthermore, Sid2p localization to the medial region suggests that Sid2p may be interacting with components of the contractile ring and septum. One putative Sid2p binding protein may be Cdc15p, or Cdc15p associated proteins, since Sid2p failed to localize to the contractile ring in *cdc15* mutants (Sparks *et al.*, 1999). Furthermore, *cdc15* mutants do not form septa, which differs from other ring mutants such as Mid1p as well as other proteins required for contractile ring assembly (Sparks *et*

al., 1999). This contrast may be due to its inability to recruit Sid2p to the ring (Sparks *et al.*, 1999).

Other proteins were also found to be regulators of the SIN pathway, and they are Etd1p, Dma1p, Clp1p/Flp1p and Nuc2p. *etd1* is an essential gene with no known resemblance to proteins found in the NCBI database (Daga *et al.*, 2005; Krapp and Simanis, 2005). Etd1p-GFP is localized to the cell cortex, enriched at the cell tips in interphase cells. In early anaphase, Etd1p is then found at the cell cortex at the medial region of the cells and in late anaphase as a tight ring before septation. HA-tagged Etd1p showed synthetic lethality with HA-tagged Cdc15p and co-immunoprecipitation experiments indicated a direct interaction between these two proteins. Etd1p resembles SIN mutants in that cells with a loss-of-function mutant of *etd1* (*etd1-1*) form a ring that fails to constrict and that later disassembles (Daga *et al.*, 2005; Krapp and Simanis, 2005). Etd1p appears to function late in the cascade as Mob1p-GFP and Sid2p-GFP failed to localize to the medial region after anaphase in *etd1-1* cells. Also, relocation of Etd1p from the medial cell cortex to the ring requires Spg1p activation. Etd1p protein levels peak during cytokinesis and Etd1p is then degraded in a ubiquitin-dependent manner by the 26S proteasome pathway. Finally, in *etd1-1* cells, the Cdc7p-GFP signal failed to be sustained suggesting that SIN activity is maintained by Etd1p. Thus, in early anaphase, Etd1p localizes to the medial region of the cell cortex (Daga *et al.*, 2005; Krapp and Simanis, 2005). Late in anaphase, SIN activity triggers the recruitment of Etd1p to the contractile ring where it associates with Cdc15p. Etd1p levels increase during contraction of the ring where the protein also accumulates. Etd1p maintains the activity of the SIN and recruits the Sid2p-Mob1p complex to the medial

region. Etd1p is then degraded, abrogating SIN signaling and allowing the cells to exit cytokinesis (Daga *et al.*, 2005; Krapp and Simanis, 2005).

Dma1p has a Forkhead phosphopeptide domain and is similar to E3 ubiquitin ligase-like RING finger proteins related to the human checkpoint protein Chfr (Murone and Simanis, 1996). Dma1p-GFP is localized to the division site in metaphase/early anaphase. In late anaphase, Dma1p-GFP was observed at both the division site and SPB, as well as on either side of the septum during septation (Murone and Simanis, 1996). It was observed that the Forkhead domain was required for Dma1p localization to both the division site and SPBs, while the RING finger domain may be required for efficient localization to the SPB (Guertin *et al.*, 2002). The Forkhead domain interacts with Sid4p in a yeast two-hybrid assay suggesting that Dma1p is associated with the SPB through Sid4p (Guertin *et al.*, 2002). Dma1p is thought to inhibit SIN signaling by promoting the dissociation of Plo1p from the SPB. This is from studies where ectopic expression of *dma1* inhibited the association of Plo1p from the SPB, and where *dma1* deletion promoted the premature association of Plo1p to the SPB (Guertin *et al.*, 2002). It is currently unknown how Plo1p acts to activate the SIN. For example, Plo1p may act to directly activate Spg1p, or may inhibit the Cdc16p-Byr4p complex allowing Spg1p-GDP to become its activated form Spg1p-GTP. The homologues of Cdc16p-Byr4p in *S. cerevisiae*, Bub2p-Bfa1p, are affected by the Polo-like kinase Cdc5p. Cdc5p phosphorylates Bfa1p, inhibiting it and allowing GTP to associate with Tem1p, the Spg1p homologue (Hu *et al.*, 2001). It is still unclear as to whether this occurs in *S. pombe*.

Clp1p/Flp1p is also a regulator of the SIN, although it is currently unknown how. Clp1p is a highly conserved protein that is not essential and deletion of this gene causes cells to enter prematurely into mitosis (Cueille *et al.*, 2001; Trautmann *et al.*, 2001). In *S. pombe*, *cdc2*, a mitotic cyclin dependent kinase required for entry to mitosis, allows mitosis to proceed by being phosphorylated on a highly conserved tyrosine residue (Tyr15) (Gould and Nurse, 1989). The protein kinase *wee1* phosphorylates Tyr15, whereas the protein phosphatase *cdc25* dephosphorylates this residue. The Clp1p protein is thought to promote tyrosine phosphorylation by the inhibition of *cdc25*, or the activation of *wee1*, or by promoting both events. The localization of Clp1p changes throughout the cell cycle. Clp1p is present in the nucleolus and on the SPBs during G1 and S phase. During early mitosis, it is then released from the nucleolus and localizes to the mitotic spindle and medial ring, and an active SIN is required for Clp1p to maintain its localization out of the nucleolus (Jin *et al.*, 2007). Clp1p also appears to be important for cytokinesis. A small percentage of *clp1* deleted cells show cytokinesis defects and *clp1Δ* cells demonstrate genetic interactions with SIN mutants. For example, in cases where the SIN is over activated, such as in cells overexpressing *spg1* or in a *cdc16* loss-of-function mutant, the cytokinesis defects are suppressed in a *clp1Δ* background (Jin *et al.*, 2007). Furthermore, Sid1p localization to the SPB is dependent on Clp1p. It is unknown how Clp1p acts to regulate the SIN, however one hypothesis is that Clp1p keeps Cdc2p activity low so that Sid1p can localize to the SPB and execute septation. The cues that release Clp1p from the nucleolus, as well as the substrates it interacts with at the SPB or medial ring are unknown (Jin *et al.*, 2007).

Nuc2p has been shown to be a negative regulator of the SIN. Nuc2p is a tetratricopeptide repeat (TPR)-domain containing subunit of the cyclosome or anaphase promoting complex (APC) (Chew and Balasubramanian, 2008). The APC is a multisubunit E3 ubiquitin ligase responsible for mitotic exit and is highly conserved. Cells overproducing Nuc2p die as elongated, multinucleate cells (Chew and Balasubramanian, 2008). At the non-permissive temperature, the *nuc2-663* strain undergoes cytokinesis in the absence of chromosome segregation. 28% of *nuc2-663* cells, at the restrictive temperature display multiple septa indicating that the cytokinetic machinery may be constitutively active in these cells (Chew and Balasubramanian, 2008). In *nuc2-663* cells, Cdc7p and Sid1p persisted at the SPBs even after the completion of cytokinesis. This is in contrast to wild-type cells, where Cdc7p and Sid1p are detected on the SPBs in cells undergoing cytokinesis, but were absent in fully septated cells (Chew and Balasubramanian, 2008). Sid2p was also persistently localized to the division plane in *nuc2-663* cells. This phenotype is similar to that of *cdc16* temperature-sensitive strains. As described previously, Cdc16p also negatively regulates the SIN and these results suggest that Nuc2p may be downregulating the SIN pathway. The *nuc2-663* strain was shown to partially degrade the mitotic B-type cyclin, Cdc13p. Cdc13p degradation, and the subsequent inactivation of the cyclin dependent kinase, Cdc2p, is sufficient for the completion of cytokinesis. Thus, it was possible that the multiseptate phenotype of *nuc2-663* cells could be due to partial proteolysis of cyclin B and/or re-entry into mitosis (Chew and Balasubramanian, 2008). To examine this, a double-mutant *nda3-KM311 nuc2-663* strain was constructed to observe the inactivation of *nuc2* after anaphase. The *nda3* gene encodes the β -subunit of the tubulin

heterodimer. The *nda3-KM311* mutant results in cold-sensitivity and lethality at 19°C. Cells containing the *nda3-KM311* mutant synchronize at metaphase due to the activation of the spindle assembly checkpoint caused by the loss of function of β -tubulin. 6 minutes after returning the cells to the permissive temperature, the microtubules are able to polymerize, and progression to chromosome segregation and mitotic exit occurs (Chew and Balasubramanian, 2008). *nuc2-663 nda3-KM311* cells formed multiple septa or deposited excessive septal material in the vicinity of the first septum. Contractile rings also formed with the septa as shown with α -Cdc4p immunostaining (Chew and Balasubramanian, 2008). *nda3-KM311* cells, which were used as a control, failed to form multiple septa. The *nuc2-663* strain was then tested for its ability to initiate septation in interphase cells. As a positive control, *cdc16-116* cells were observed in parallel. The *nuc2-663* and *cdc16-116* strain were arrested in S phase with hydroxyurea and then shifted to the non-permissive temperature to inactivate the *nuc2* and *cdc16* genes. Inactivation of *cdc16* caused the cells to septate during interphase (Chew and Balasubramanian, 2008). Inactivation of *nuc2* however, did not cause cells to initiate septation in interphase. Thus, *nuc2* may play an important role in preventing additional rounds of cytokinetic events following septation. Other subunits of the APC such as Cut9p and Lid1p failed to produce a multiseptate phenotype suggesting that the inhibition of inappropriate cytokinesis by Nuc2p might not require additional subunits of the APC (Chew and Balasubramanian, 2008). The authors then constructed a strain in which the endogenous *nuc2* promoter was replaced with the *nmt1* promoter (*nmt1-nuc2*) (Chew and Balasubramanian, 2008). Using these cells, the formation of the contractile ring was followed using Rlc1p-GFP. In *nmt1-nuc2* cells,

Rlc1p-GFP formed rings, but the rings failed to constrict and collapsed in late anaphase (Chew and Balasubramanian, 2008). Cdc7p and Sid1p were also absent from the SPBs compared to wild-type cells which showed that 80% of the cells contained Cdc7p-GFP and Sid1p-GFP at the SPBs. The localization of Sid4p, Cdc11p and Spg1p was unaltered. Thus, overexpression of *nuc2* affects the recruitment of Cdc7p and Sid1p to the SPB. Spg1p binds to Cdc7p, recruiting it to the SPB during mitosis. Cdc7p binds preferentially to Spg1p-GTP, the active form of the GTPase. Since Cdc7p localization to the SPB was diminished in *nmt1-nuc2* cells, an excess of *nuc2* may affect the interaction between Cdc7p and Spg1p-GTP. To see if this was the case, *nuc2* was overexpressed in cells containing Spg1p-GFP and Cdc7p-3HA, and co-immunoprecipitation experiments were performed. In these cells, very little Spg1p-GFP pulled-down Cdc7p-3HA (Chew and Balasubramanian, 2008). Thus, the binding of Cdc7p-3HA and Spg1p-GFP was interrupted in cells overexpressing *nuc2*. Two hypotheses were raised on why the interaction was severely disabled: (A) a putative guanine nucleotide exchange factor (GEF) could be inactivated; or (B) the two-component GAP, Byr4p-Cdc16p may be activated. Since a GEF for the Spg1p GTPase has not yet been discovered in *S. pombe*, the potential activation of the two-component GAP was tested. Here, *nmt1-nuc2 cdc16-116* cells were assayed for their ability to localize Cdc7p to the SPB. In these cells, Cdc7p was recruited and maintained on the SPB, in contrast to *nmt1-nuc2* cells alone where very little Cdc7p was recruited to the SPB (Chew and Balasubramanian, 2008). Thus, it is possible that increased *nuc2* leads to the activation of the Byr4p-Cdc16p complex causing defects in septation. Finally, Nuc2p function was found to be independent of Dma1p function. No differences in

septation were observed in cells overproducing *nuc2* and lacking *dma1*. Thus, Dma1p and Nuc2p inhibit the SIN by different mechanisms.

In conclusion, Spg1p is the molecular switch driving actomyosin ring contraction and septation from the nucleus (Figure 1.5). Spg1p is negatively regulated by the two component GAP, Byr4p and Cdc16p. Asymmetric distribution of Byr4p and Cdc16p at the onset of anaphase causes the active form of Spg1p (Spg1p-GTP) to recruit Cdc7p on the opposite SPB to Byr4p and Cdc16p. Cdc11p and Sid4p are thought to act as scaffold proteins offering a platform for the binding of Sid1p-Cdc14p and Sid2p-Mob1p complex and triggering its localization to the medial ring, where Sid2p may phosphorylate other proteins in the medial ring. Phosphorylation of certain medial ring components, such as Cdc15p, may then result in actomyosin ring contraction and septation. However, this model is complicated by the observed localization of the Sid2p-Mob1p complex to both SPBs. Moreover, it is still unclear how Plo1p may act to regulate the Spg1p switch.

It is apparent that mitotic exit and cytokinesis are coupled by proteins in the SIN and three proteins that provide evidence for this are: Plo1p (which requires the action of Cdc25p and Cdc2p-Cdc13p to become activated and requires the SIN for downregulation) (Tanaka *et al.*, 2001); Cdc16p (which is required to maintain Cdc2p kinase activity during mitosis) (Fankhauser *et al.*, 1993); and Cdc11p (which is hyperphosphorylated at anaphase) (Krapp *et al.*, 2003) (Figure 1.5).

1.1.3.5. Membrane trafficking and cell separation

1.1.3.5.1. Proteins involved in membrane trafficking are required for cytokinesis

Membrane expansion is required at the medial region for cytokinesis to occur. There is very little known about this process in *S. pombe*, since most of the knowledge in this area has come from research in plants and animals. Electron microscopy has however revealed that vesicles are targeted to the septum late in cytokinesis in fission yeast, likely to build plasma membrane and cell wall (Kanbe *et al.*, 1989). The actomyosin ring must be tightly associated with the plasma membrane and interactions between the ring and plasma membrane at the cleavage site are required to co-ordinate the contractile and exocytic events necessary for cytokinesis to occur. In mammalian cells during cytokinesis, membrane may be acquired from the poles of the cell or by membrane addition from the Golgi or recycling endosomes (REs) to the medial region (Strickland and Burgess, 2004, for review).

In *S. pombe*, the amphiphilic dye FM4-64 has been used as a marker for endocytic events. FM4-64 is endocytosed and transported to the vacuolar membrane. Time course experiments showed that the dye was taken up first at the old end of fission yeast cells, and once NETO began, to the new end (Gachet and Hyams, 2005). This was corroborated by GFP-Syb1p staining. This protein will be discussed in greater detail below. These areas of endocytosis also co-localized with actin patches and were dependent on the presence of actin through studies with latrunculin A and B, potent actin depolymerizing agents. Furthermore, endocytosis was observed at the medial region. Studies of FM4-64 staining in *cdc7-24*, *sid1-125* and *sid2-250* mutants revealed that there was no endocytosis at the equator at the restrictive temperature whereas endocytosis at the cell tips remained intact (Gachet and Hyams, 2005). Thus, endocytosis at the medial region requires the SIN pathway. The same was observed in

cells treated with brefeldin A (BFA). BFA is a potent inhibitor of the secretory pathway by affecting the activation of Sec7p-type GTPase exchange factors (GEFs) that catalyze the activation of the small GTPase Arf1 in mammalian and yeast cells (Nebenfuhr *et al.*, 2002). Arf1, at the Golgi, recruits coat proteins such as COPI and clathrin (via the adapter complex AP-1) to membranes resulting in the formation of transport vesicles (Scales *et al.*, 2000). Thus, BFA disrupts the formation of transport vesicles from the Golgi thereby blocking secretion.

The study of several proteins involved in membrane trafficking in fission yeast have also provided clues into the importance of this event during cytokinesis. These proteins are the synaptobrevin homologue, Syb1p, the SNAP-25 homologue, Sec9p, the μ 1 subunit of the clathrin-associated adaptor protein 1 (AP-1) complex, Apm1p, the Rab11 homologue, Ypt3p, and the phosphatidylinositol/phosphatidylcholine transfer protein (PITP), Spo20p.

Intracellular membrane trafficking is mediated by a conserved family of membrane associated proteins known as the SNARES (Jahn and Sudhof, 1999). SNARES are classified into several families: synaptobrevin (v-SNARES), syntaxin (t-SNARES) and SNAP-25 (s-SNARES or peripheral SNARES) (Jahn and Sudhof, 1999). These proteins form a complex of fusion machinery at the target membrane which triggers membrane fusion. Synaptobrevin forms an alpha helical bundle with syntaxin and SNAP-25 (Jahn and Sudhof, 1999). *S. pombe* Syb1p contains a transmembrane domain in its C-terminal region, a basic region proximal to the transmembrane domain, a conserved coiled-coil domain and a variable N-terminal region (Edamatsu and Toyoshima, 2003). *syb1* is an essential gene and is 30-35% identical to human

synaptobrevins. *syb1* null mutants arrested as short cells suggesting a role for Syb1p in cell elongation. GFP-Syb1p was located on vesicle-like structures distributed throughout the cytoplasm in both dividing and interphase cells. Also, GFP-Syb1p localized to the cell ends in interphase cells and to the medial region in dividing cells (Edamatsu and Toyoshima, 2003). GFP-Syb1p localization to the medial region was dependent on actin structures since latrunculin A removed the distribution of Syb1p from the medial region. Also, GFP-Syb1p failed to localize to the medial region in *myo52Δ* cells, suggesting that Myo52p transported Syb1p to the medial region. Myo52p is one of two type V myosins in *S. pombe* and is required for the transport of vesicles in cells. This effect was dependent on Myo52p because Syb1p localization to the medial region was not affected in *myo51Δ* cells. Myo52p is localized to the division site and *myo52Δ* cells accumulate a large number of vesicles in the cytoplasm suggesting that Myo52p is involved in targeted vesicle transport to the division site (Win *et al.*, 2001; Mulvihill *et al.*, 2001; Mulvihill *et al.*, 2006). This vesicle transport appears to be important for septation since in the *myo52* mutant, contractile rings form normally, but septation is greatly delayed. In mammalian cells, synaptobrevin binds directly to the tail region of rat brain myosin V (Ohyama *et al.*, 2001). Purified synaptobrevin did not bind to the tail of Myo52p suggesting an alternate mechanism in fission yeast (Edamatsu and Toyoshima, 2003). Once Syb1p formed at the medial region, the removal of actin or microtubules did not disrupt its localization, suggesting that Syb1p is anchored at the medial region by another factor besides actin or microtubules. In mammalian cells, synaptobrevin was shown to bind septins directly suggesting that Syb1p may be anchored by septin filaments at the division site mediated

by syntaxin (Beites *et al.*, 1999). No loss-of-function alleles of this protein were generated, however, its localization to the medial region suggest a role in cytokinesis.

Sec9p is the sole SNAP-25 homologue in *S. pombe* and has 41% similarity with human SNAP-25. SNAP-25 is an s-SNARE involved in the fusion of vesicles with the target membrane (Sollner *et al.*, 1993; Rothman, 1994; Rothman and Warren, 1994; Pelham, 1999; Jahn *et al.*, 2003). Studies of the temperature-sensitive *sec9-10* mutant show that 43% of the cells at the restrictive temperature of 34°C had a single septum, whereas 4% exhibited multiple septa (Nakamura *et al.*, 2005). These levels are elevated from wild-type levels of septation, which range from 10% to 15% in an asynchronous culture. This implies that Sec9p is important for cytokinesis. Thus, aberrant membrane trafficking affects the termination of septation and cell separation. In mammalian cells, SNAP-25 forms a complex with syntaxin 1 and synaptobrevin. The syntaxin 1 homologue in *S. pombe* is *psyl*. Increased expression of *psyl* suppressed the *sec9-10* mutant phenotype at 34°C (Nakamura *et al.*, 2005). However, increased expression of *syb1* could not suppress the *sec9-10* mutant phenotype. This suggests that *psyl* genetically interacts with *sec9*. Further studies to elucidate the role of *syb1* in this complex, is required to determine if *syb1* acts in a manner similar to synaptobrevin in mammalian cells.

Apm1p is the μ 1 subunit of the clathrin-associated AP-1 complex. AP-1 mediates protein sorting at the *trans*-Golgi and the μ subunits have been implicated in cargo selection (Boehm and Bonifacino, 2001). Apm1p is 62% similar to human μ 1A (Kita *et al.*, 2004). The *apm1* gene is not essential at lower temperatures (i.e., 25°C), but is essential at high temperatures (i.e., 36°C). Cells deleted for *apm1* showed Golgi

defects and Berkeley bodies (disrupted Golgi structures) which became more severe upon shift to a higher temperature. These cells also displayed a decrease in the secretion of acid phosphatase compared to wild-type cells at 27°C (Kita *et al.*, 2004). GFP-Syb1p failed to localize to the cell tips and to the medial region in cells deleted for *apm1* suggesting that a defect in AP-1 dependent membrane traffic affects Syb1p localization. Apm1p-GFP localized to the medial region and SPBs in dividing cells. Apm1p also co-localized with FM4-64 staining representing Golgi/endosomal compartments (Kita *et al.*, 2004). Cells deleted for *apm1* at 36°C showed an increase in the frequency of septated cells (38%). The authors did not comment on this, but the septa appeared thicker than in wild-type cells. Furthermore, vacuole defects were observed in cells deleted for *apm1*. For example, *apm1Δ* vacuoles remained small after a 90 minute incubation in water, whereas in wild-type cells, the vacuoles were large due to vacuole fusion processes (Kita *et al.*, 2004). The maturation of the vacuolar carboxypeptidase Y (CPY) protein and endocytosis remained unaffected suggesting that vacuolar protein sorting and secretion was normal in these cells. Finally, *apm1Δ* cells displayed cell wall defects such as sensitivity to β-glucanase treatment and restored growth at 37°C in the presence of increased osmolarity (1.2 M sorbitol).

Ypt3p is the homologue of Rab11 in mammalian cells (69% identical) and Ypt31p (62% identical) and Ypt32p (63% identical) in budding yeast (Cheng *et al.*, 2002). It is an essential gene, thus functional studies were carried out with the *ypt3-i5* mutant. The *ypt3-i5* mutant has an R29H mutation which is temperature-sensitive and lethal at 36°C. *ypt3-i5* cells grown at the restrictive temperature had unusually thick septa that were brightly stained with calcofluor and electron microscopy showed that

the septa were indeed unusually thick (Cheng *et al.*, 2002). This phenotype was very similar to that of *apm1* deleted cells. The localization of Ypt3p was observed by ectopically expressing Ypt3p-GFP under the *nmt1* promoter. GFP-Ypt3p was observed at the cell tips and medial region, as well as to punctate structures throughout the cell cycle (Cheng *et al.*, 2002). The Ypt3p protein did not colocalize with Gma12p-HA, which is a Golgi associated protein, indicating that Ypt3p is not Golgi associated. The localization of GFP-Ypt3-R29H was aberrant in that there was no longer Ypt3p localization to the cell tips or medial region accounting for the loss-of-function of this allele (Cheng *et al.*, 2002). Addition of 1.2 M sorbitol to the growth medium complemented the temperature-sensitive phenotype of *ypt3-i5* and *ypt3-i5* cells lysed faster in the presence of β -glucanase indicating defects in the cell wall. Like the *apm1* deletion mutant, *ypt3-i5* displayed vacuole fusion defects and abnormal Golgi structures such as Berkeley bodies.

Finally, the PITP Spo20p was observed to be involved in cytokinesis since cells carrying the *spo20-KC104* temperature-sensitive mutation at 37°C showed that 60% of cells had a septum and 8% had multiple septa (Nakase *et al.*, 2001). Spo20p is homologous to Sec14p in *S. cerevisiae*. *spo20* is an essential gene and the *spo20-KC104* mutation encoded a G275D mutation, which was temperature-sensitive and lethal at 35°C (Nakase *et al.*, 2001). Electron microscopy of cells carrying the G275D mutant at the restrictive-temperature showed an accumulation of Golgi cisternae. Spo20p-HA localized to the cell tips in interphase cells and to the medial region in late mitotic cells. For example, after the ring constricted, Spo20p localized to the medial region. The actin cytoskeleton is required for Spo20p to localize to the medial region

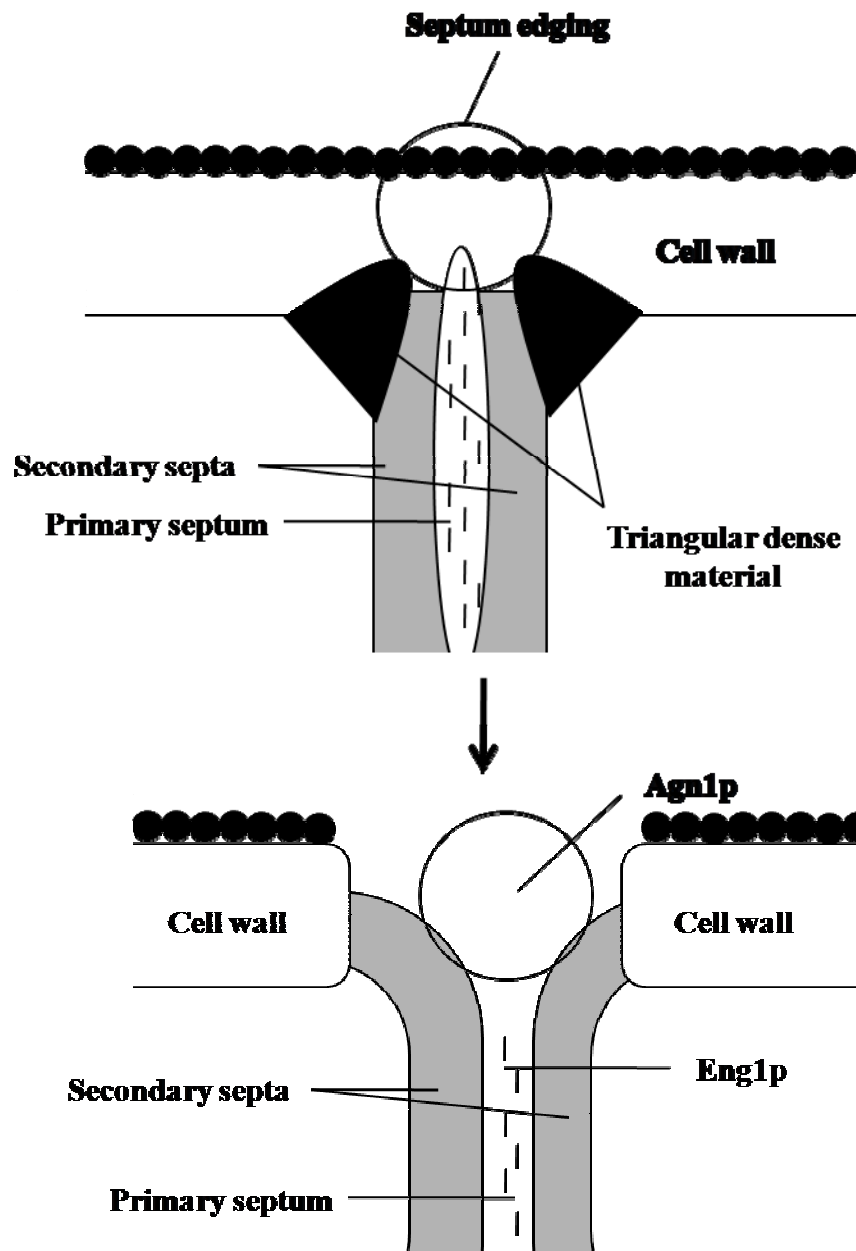
and cell tips (Nakase *et al.*, 2001). Thus, consistent with membrane trafficking being important for the final steps of cytokinesis in animal cells, membrane trafficking appears to be important for septation in fission yeast. In depth analysis of the Syb1p, Apm1p, Sec9p, Ypt3p and Spo20p mutants for effects on contractile ring formation should be performed to determine if membrane trafficking events are required for contractile ring assembly/maintenance/stability.

1.1.3.5.2. Membrane trafficking is required for cell-cell separation

Cytokinesis in fission yeast is completed when the two cells become membrane delimited and when the daughter cells physically separate by the dissolution of the primary septum and septum edging (Figure 1.6). *S. pombe* is a yeast that has a cell wall, thus new cell wall material must be targeted and assembled at the cell equator and then hydrolyzed causing the separation of the two daughter cells. Cell separation is achieved by the digestion of the primary septum and surrounding cell wall mediated by the Eng1p (endo- β -1,3) and Agn1p (endo- α -1,3) glucanases (Martin-Cuadrado *et al.*, 2003). These enzymes are targeted to the septum for cell separation and likely diffuse through the septum to their polysaccharide target. During septation, a separation septum is built as an extension of the cell wall and has a three layered structure (Humbel *et al.*, 2001). The central layer (primary septum) consists mainly of linear β -1,3-glucan synthesized by the Cps1p/Bgs1p protein which is a component of the β -1,3-glucan synthase complex (Le Goff *et al.*, 1999; Liu *et al.*, 2000). The primary septum is required to avoid cell lysis during the progression of cytokinesis. Linear β -1,3-glucan is unique to the primary septum and is not found in the cell wall. The outer layers,

Figure 1.6: Cell separation in *S. pombe*.

The primary septum is composed uniquely of β -1,3-glucan and is hydrolyzed by the Eng1p endo-(1,3)- β -glucanase. The secondary septa, which is composed of β -1,6-branched β -1,3-glucan, β -1,6-glucan and α -1,3-glucan, is hydrolyzed by Agn1p, an endo-(1,3)- α -glucanase. Agn1p hydrolyzes the septum edging, which is a region of the cell wall adjacent to the primary septum. Figure adapted from Dekker *et al.*, 2004.



consisting of the secondary septa, are mainly composed of β -1,6-branched β -1,3-glucan and β -1,6-glucan (Humbel *et al.*, 2001). Cell separation not only involves the dissolution of the primary septum through the Eng1p (1,3)- β -glucanase, but the dissolution of the septum edging consisting of β -1,6-branched β -1,3-glucan and α -1,3-glucan which is absent from the primary septum. The Agn1p endo-1,3- α -glucanase dissolves the septum edging (Dekker *et al.*, 2004) (Figure 1.6).

The cell wall is composed of glucan and galactomannan polysaccharides. It is composed of 9-14% α -galactomannan, 18-28% α -1,3-glucan, 42% β -1,3-glucan, 2% β -1,6-glucan and small amounts of chitin (Osumi *et al.*, 1998). The presence of chitin in the cell wall of *S. pombe* has been controversial. However, the presence of glucosamine, a monomeric unit of chitin, chitin synthase activity, and a chitin synthase gene homologue, have been found in *S. pombe* indicating that chitin is present in the cell wall. The *S. pombe* cell wall has a 3 layered structure with an outer α -galactomannan layer. The β -1,6-branched β -1,3-glucan radiates from the cell membrane to the α -galactomannan layer. The β -1,6-glucan polysaccharide is closer to the α -galactomannan layer than to the cell membrane.

Once the septum has been synthesized, temporally and spatially controlled recruitment of the glucanases to the septum cell wall is required to degrade the primary septum accompanied by the local degradation of adjacent regions of the cell wall (Martin-Cuadrado *et al.*, 2003). At least two levels of regulation are required for the controlled dissolution of the septum: the targeted secretion of hydrolytic enzymes and the cell cycle regulated expression of cell separation genes.

There are mutants that are defective for cell separation, some of which include components of the exocyst complex (*sec6*, *sec8*, *sec10*, and *exo70*) (Wang *et al.*, 2002). The exocyst is a multisubunit complex that is required for the targeting and fusion of Golgi-derived vesicles to the plasma membrane (Wang *et al.*, 2002; Lipschutz and Mostov, 2002). The exocyst is regulated by the Ras superfamily of small GTP-binding proteins. Mutants of the exocyst are capable of polarized growth, cell surface expansion and division assembly, but are specifically defective in the dissolution of the septum cell wall material and cell separation (Wang *et al.*, 2002). All components of the exocyst are essential except for *exo70*. Immunoprecipitation experiments showed that Sec6p, Sec8p, Sec10p and Exo70p form a complex *in vivo*. Sec8p-GFP localizes to the cell tips in interphase cells. In early mitotic cells, tip localization was absent and Sec8p-GFP was seen as a ring in the medial region of the cell that resembled the contractile ring. In late mitotic cells, after the contractile ring constricted, Sec8p-GFP was detected as double rings. The same localization was observed for Sec6p-GFP, Sec10p-GFP and Exo70p-Myc. Sec10p-GFP did not form rings in *cdc12-112* or *cdc15-140* mutants, thus, assembly of the exocyst into rings at the medial region depends on proteins essential for actin patch mobilization and contractile ring assembly.

Most recently, the Exo70p subunit of the exocyst was shown to bind PtdIns(4,5)P₂ in *S. cerevisiae* (He *et al.*, 2007). Co-sedimentation assays showed that the C-terminal region of Exo70p (amino acids 323-623), which is highly conserved from yeast to mammals, binds to artificial large unilamellar vesicles containing 5% PtdIns(4,5)P₂ and 20% phosphatidylserine. This composition mimics biological membranes. Also, using a Ras-rescue assay, Exo70p was shown to be targeted to the

plasma membrane. The Ras-rescue assay is used to identify proteins that bind strongly to PtdIns(4,5)P₂ in the inner leaflet of the plasma membrane in yeast. *CDC25* encodes a Ras guanine nucleotide exchange factor (GEF) that activates Ras at the plasma membrane and is essential for survival. Co-expression of a fusion protein with an activated form of Ras (RasQ61L) with proteins that target RasQ61L to the plasma membrane, rescues the *CDC25* temperature-sensitive phenotype at 37°C.

The targeting of exocyst subunits to the plasma membrane requires PtdIns(4,5)P₂ because disruption of the synthesis of this lipid through the loss-of-function of Mss4p, results in the loss of localization of the exocyst subunits to the bud tip membrane (He *et al.*, 2007). Mss4p is a PtdIns4P 5-kinase that is involved in generating PtdIns(4,5)P₂ at the plasma membrane. Both Exo70p and Sec3p (another component of the exocyst complex) associate stably with the bud tip membrane suggesting that both Exo70p and Sec3p may act in concert to mediate anchorage of the exocyst complex to the plasma membrane. Mutants of Exo70p that could not bind PtdIns(4,5)P₂ were expressed in a *sec3* background where the N-terminal membrane targeting domain was deleted (*sec3ΔN*). As expected, the exocyst subunits were highly delocalized in these cells. Also, exocytosis of Bgl2p, an endoglucanase that is involved in cell wall remodeling during daughter cell growth, was greatly affected in these cells. Thus, membrane targeting of the exocyst subunits Exo70p and Sec3p, mediated through interactions with PtdIns(4,5)P₂, is essential for functional exocytosis.

At the restrictive temperature, *sec8-1* cells showed an accumulation of vesicles around the septa as visualized by electron microscopy. Thus, targeting of vesicles appears to be normal, but docking and/or fusion with the plasma membrane is impaired.

This was corroborated by the finding that acid phosphatase secretion was aberrant in *sec8-1* cells compared to wild-type cells. Since mutants of the exocyst failed to separate, it was proposed that these mutants were defective for the delivery of important hydrolytic enzymes required to dissolve the primary septum and septum edging, possibly the Eng1p and Agn1p endo-glucanases (Wang *et al.*, 2002).

Indeed, studies showed that the exocyst *exo70* and *sec8* genes are required for the proper localization of Eng1p-GFP to the septum (Martin-Cuadrado *et al.*, 2005). In *exo70Δ* and *sec8-1* cells, Eng1p-GFP appeared as a disc, instead of the normal ring-like structure at the septum observed in wild-type cells. Fluorescence recovery after photobleaching (FRAP) experiments indicated that the recovery of fluorescence of Eng1p-GFP at the medial region was slower in *sec8-1* cells at 25°C. The same was observed for Agn1p-GFP (Martin-Cuadrado *et al.*, 2005).

Septins are a conserved family of GTP-binding proteins which function in cytokinesis (Versele and Thorner, 2005 for review). They do so by regulating secretion and membrane deposition at the division site (An *et al.*, 2004). Septins in animal cells associate with components of the secretory pathway such as the exocyst complex and the SNARE protein syntaxin (Hsu *et al.*, 1998; Beites *et al.*, 1999). N-terminal to the GTP-binding domain is a phosphoinositide binding motif that binds plasma membrane phosphatidylinositol 4,5 biphosphate (PtdIns(4,5)P₂). The interaction of septins with PtdIns(4,5)P₂ at the plasma membrane is important for the maintenance of proper septin organization *in vivo* in yeast and animal cells (Zhang *et al.*, 1999; Casamayor and Snyder, 2003). *S. pombe* has seven septins, four of which are localized to the division plane and three of which are involved in sporulation and meiosis (Berlin *et al.*, 2003).

The four septins involved in cell division are named Spn1p, Spn2p, Spn3p and Spn4p (An *et al.*, 2004). *S. pombe* septin deletion mutants are viable, but are defective in cell separation forming chains of cells (Longtine *et al.*, 1996). Indeed, a quadruple deletion of all the septins produces the same phenotype as the single deletion mutants (An *et al.*, 2004; Berlin *et al.*, 2003; Tasto *et al.*, 2003). GFP tagged septins localize to the contractile ring late in cytokinesis just before the spindle reaches its full length in mitosis and before contraction of the acto-myosin ring begins (Wu *et al.*, 2003). During acto-myosin ring contraction, and during septum cell wall formation, the septin ring splits into two, flanking both sides of the septum, and later dissolves away (Longtine *et al.*, 1996). The septin ring does not constrict with the acto-myosin ring during cytokinesis (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Unlike the septins in budding yeast, fission yeast septins are not essential for viability (Longtine *et al.*, 1996).

A protein required for septin ring formation is the anillin-like protein Mid2p. The term anillin-like refers to its sequence similarity to anillin in mammalian cells (Wu *et al.*, 2003). Mid2p does not contain an actin binding domain unlike animal anillin (Versele and Thorner, 2005). The Mid2p homologue in *S. cerevisiae*, Bud4p, associates with the IQGAP homologue, Iqg1p (Osman *et al.*, 2002). Mid2p is also related to the Mid1p anillin-like protein in *S. pombe* involved in division site placement. Like the septins and *mid1*, *mid2* is not essential for viability. Also, like the septin deletion mutants, *mid2* deletion mutants form chains of cells. In an asynchronous culture of *mid2Δ* cells, 66% contained septa, <5% had 2-3 septa and grew in short chains of cells whereas wild-type cells had a septation index of 16%. Assembly and contraction of the

contractile ring in *mid2Δ* cells remained unchanged from wild-type cells suggesting that Mid2p is not required for contractile ring formation.

Mid2p is required for the proper organization of the septin ring at the division plane (Berlin *et al.*, 2003). Cells deleted for *spn4* had a similar septation index as cells deleted for *mid2* and double-mutant cells showed a similar phenotype suggesting that they acted in the same pathway. In *mid2Δ* cells, Spn4p-GFP did not form double rings, but formed an aberrant double disc-like structure. Thus, Mid2p has a specific role in maintaining the organization of the septin ring during contraction. This was corroborated by FRAP experiments that showed that Spn4p-GFP in *mid2Δ* cells recovered 30-fold faster than in wild-type cells, suggesting that Mid2p is required to stabilize the septin ring. Mid2p colocalizes with the septins in single and double rings during mitosis. Septins localize normally in *mid2Δ* cells, but Mid2p does not localize normally in *spn4Δ* cells. This indicates that Mid2p associates with the septin ring slightly later in the cell cycle. Thus, it is thought that Mid2p is required for maintaining stable septin rings at the end of cytokinesis to facilitate cell separation (Berlin *et al.*, 2003).

The Mid2p protein has a PH domain at its C-terminus. The Mid2p PH domain was disrupted through the addition of the KanMX or YFP-KanMX gene cassette to create a truncation mutant (Berlin *et al.*, 2003). Interestingly, the Mid2p PH domain was required for Mid2p function and localization. *mid2-PHA* cells had the same cell-cell separation defect as *mid2Δ* cells. Also, the *mid2-PHA*-YFP cells exhibited diffuse staining indicating that the protein was not localized properly (Berlin *et al.*, 2003). The Mid1p and Mid2p proteins do not have overlapping functions. The double-mutant

versions were not more severe than the single mutants and Mid1p was properly localized in *mid2Δ* cells and Mid2p was properly localized in *mid1Δ* cells (Berlin *et al.*, 2003).

The Eng1p and Agn1p endo-glucanases localize to the septum late in cytokinesis as a ring-like structure. Components of the exocyst (Sec6p, Sec8p, Sec10p and Exo70p), as well as septins, and Mid2p, are required for the proper recruitment of Eng1p and Agn1p to the septum (Martin-Cuadrado *et al.*, 2005). In *mid2Δ* and *spn1Δ* cells, Eng1p-GFP was observed as a diffuse disc-like structure rather than the ring-like structure observed in wild-type cells, and FRAP experiments indicated that in *mid2* and septin deleted cells, Eng1p-GFP recovery was 3-fold slower than in wild-type cells. Thus, it is hypothesized that the targeting of Eng1p and Agn1p requires normal septin rings which act as positional markers to recruit secretory vesicles via the exocyst (Martin-Cuadrado *et al.*, 2005). In *mid2* deletion mutants, abnormal septin rings persist causing aberrant localization of the secretory vesicles carrying Eng1p and Ang1p, and aberrant cell-cell separation (Martin-Cuadrado *et al.*, 2005).

To ensure the temporal and spatial regulation of hydrolytic enzyme function during cytokinesis, there is transcriptional regulation of both of the endo-glucanase genes. Mutation of the transcription factor Ace2p interferes with cell separation (Ribar *et al.*, 1999; Alonso-Nunez *et al.*, 2005). Ace2p induces the expression of several genes required for cell separation at septation, some of which are *agn1*, *eng1* and *mid2* (Alonso-Nunez *et al.*, 2005). Expression of these genes occurs late in mitosis. Cells deleted for Ace2p are multinucleate and hyphate resembling filamentous fungi (Martin-Cuadrado *et al.*, 2003; Tasto *et al.*, 2003).

Other genes required for cell separation include calcineurin (*ppb1*) (Yoshida *et al.*, 1994), a MAPK (*pmk1*) (Toda *et al.*, 1996), a MAPK phosphatase (*pmp1*) (Sugiura *et al.*, 1998), the transcription factor *sep1* (Ribar *et al.*, 1999), Rho GTPases (*rho3* and *rho4*) (Wang *et al.*, 2003; Santos *et al.*, 2003) and subunits of the transcriptional mediator complex (*sep10*, *sep11* and *sep15*) (Szilagyi *et al.*, 2002; Zilahi *et al.*, 2000).

1.1.3.6. Cdc4p

Of all the proteins involved in cytokinesis, the focus of my research was Cdc4p. Cdc4p was first identified in a screen for *cdc* mutants (Nurse *et al.*, 1976). The gene was cloned by McCollum *et al.* (1995) and found to be related to small EF-hand proteins such as calmodulin, troponin C and myosin light chains (McCollum *et al.*, 1995). In 1999, the protein was identified as a myosin essential light chain (Naqvi *et al.*, 1999), and in 2001, a yeast two-hybrid screen with Cdc4p suggested the possibility of an interaction with Pik1p and a Vps27p-like protein involved in phosphatidylinositol (PtdIns) metabolism and vesicle trafficking, respectively. This suggested that Cdc4p may have multiple functions aside from that of a myosin essential light chain.

1.1.3.6.1. Cdc4p is a myosin essential light chain

Cdc4p is a 141 amino acid protein of molecular weight 16 kDa (McCollum *et al.*, 1995). It is essential for fission yeast cell viability and is a component of the contractile ring (McCollum *et al.*, 1995). The *cdc4* gene is located on chromosome I, and the genomic sequence contains 4 introns (Wood *et al.*, 2002). Microarray data suggests that *cdc4* is transcriptionally regulated during the cell cycle by the Ace2p

transcription factor (Rustici *et al.*, 2004). Transcription of the *cdc4* gene falls into the group of transcriptionally regulated genes whose transcript levels peak at around anaphase, cytokinesis or entry into G1. *cdc4* is not transcriptionally regulated in response to environmental stress, prior to mating, or in response to pheromones (Mata *et al.*, 2006; Chen *et al.*, 2003; Mata *et al.*, 2002).

The EF-hand Ca^{2+} -binding motifs constitute a large and functionally diverse protein family (Gifford *et al.*, 2007). The EF-hand has a helix-loop-helix secondary structure which binds Ca^{2+} or Mg^{2+} ions. EF-hands tend to occur in pairs which form an independent domain so that most family members contain two, four or six EF-hands. One well characterized EF-hand protein is calmodulin which has a structure similar to Cdc4p. Like calmodulin, Cdc4p is a dumbbell shaped protein, with two globular domains connected by a flexible linker (Figure 1.7) (Slupsky *et al.*, 2001). In calmodulin, each globular domain carries an EF-hand pair that each binds to one calcium ion. Cdc4p, unlike calmodulin, has one out of four putatively functional Ca^{2+} -binding sites, based on the Cdc4p sequence, but did not bind Ca^{2+} *in vitro* (Slupsky *et al.*, 2001). Biophysical studies of Cdc4p have shown that aspects of Cdc4p structure allow it to assume different conformations leading to the suggestion that Cdc4p may be involved in multiple interactions (Escobar-Cabrera *et al.*, 2005).

Several temperature-sensitive mutants of *cdc4* have been isolated. At the restrictive temperature, *cdc4* mutants are elongated and multinucleate with 2 to 4 nuclei (Figure 1.7) (McCollum *et al.*, 1995). Mutants of *cdc4* also display disrupted contractile rings and septa. The *cdc4* deletion mutant is different than the conditional *cdc4* alleles in that the cells exhibit no contractile rings, but abnormal septal deposits

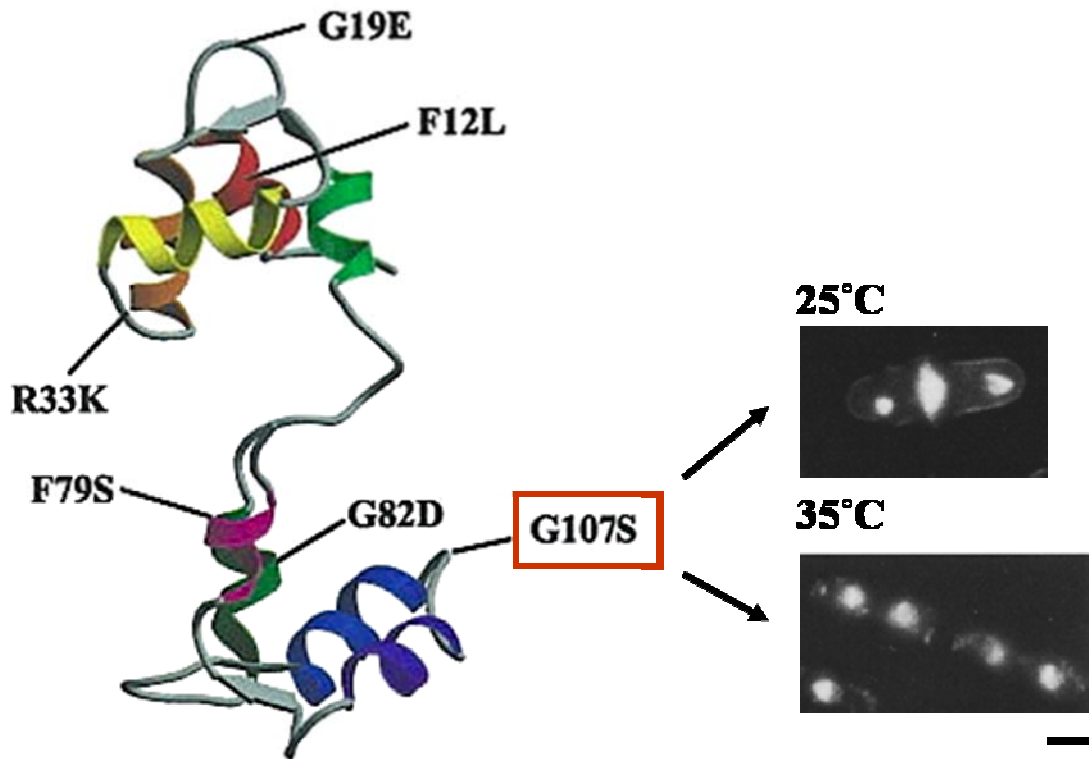


Figure 1.7: The protein structure of *S. pombe* Cdc4p and location of six temperature-sensitive mutations causing failure of cytokinesis.

Cdc4p is an EF-hand protein composed of two globular domains connected by a flexible linker (protein structure, 1GGW; Slupsky *et al.*, 2001). The missense mutations of six *cdc4* temperature-sensitive strains are as indicated. One of the alleles with the most penetrant phenotype is the *cdc4*^{G107S} allele. Cells carrying this allele and stained for nuclei (DAPI) and septa (calcofluor) at both the permissive (25°C) and non-permissive (35°C) temperatures are shown (micrometer bar represents 5 μ m). At 35°C, cells are elongated and multinucleate. Images of the *cdc4*^{G107S} allele are taken from McCollum *et al.*, 1995.

(McCollum *et al.*, 1995). The ring is thought to guide the formation of the septum. Thus, it is possible that the abnormal septal deposits may be an indirect result of aberrant, or no, contractile ring formation. These results suggest that Cdc4p is involved in actomyosin ring assembly and is a critical component of the actomyosin ring. Cdc4p is phosphorylated *in vivo* on serine 2 or 6, but not on both (McCollum *et al.*, 1999a). Cells expressing Cdc4p carrying mutations on either serine 2 or 6 were able to grow and divide normally indicating that Cdc4p phosphorylation is non-essential under normal conditions. Furthermore, the Cdc4p phosphorylation state did not affect the timing of cytokinesis or its interaction with Myo2p.

Immunostaining cells with antibodies generated against Cdc4p revealed that Cdc4p is localized to the cytoplasm in interphase cells and to the contractile ring early in mitosis (McCollum *et al.*, 1995). Cdc4p containing rings shrank co-incident with acto-myosin ring contraction and with the completion of contraction to a dot in the medial region of the cell. After the completion of cytokinesis, Cdc4p staining became diffuse and the protein was presumed to be cytoplasmic (McCollum *et al.*, 1995).

One function of Cdc4p is that of a myosin essential light chain. Cells deleted for the first and second IQ motifs in the neck region of Myo2p reveal that Cdc4p interacts strongly with the first IQ-domain and weakly to the second IQ-domain (Table 1.1) (Naqvi *et al.*, 1999; D'Souza *et al.*, 2001). Cdc4p was also found to act as myosin light chain on the other type II myosin in *S. pombe*, Myp2p/Myo3p (Table 1.1) (Motegi *et al.*, 2000). The myosin regulatory light chain (Rlc1p) was observed to bind exclusively to the second IQ-motif, the canonical binding site for the myosin II regulatory light chain (RLC) (Naqvi *et al.*, 2000). The first IQ-domain is the canonical

Table 1.1.Summary of myosins in *S. pombe* and their determined interactions with Cdc4p

Myosin	Type	Function	Interacts with Cdc4p?	References
Myo1p	I	Involved in maintaining sterol-rich membrane domains; binds to Arp2/3 complex and stimulates its actin nucleation activity	unknown	Lee <i>et al.</i> , 2000; Takeda and Chang, 2005
Myo2p	II	Molecular motor for contractile ring assembly and contraction	Yes Through Co-IP	Naqvi <i>et al.</i> , 1999; Motegi <i>et al.</i> , 2000; D'Souza <i>et al.</i> , 2001
Myp2p/Myo3p	II	Unconventional type II myosin; localizes to contractile ring late in cytokinesis and is required for cytokinesis at low temperatures and stressful conditions	Yes Through Co-IP	Motegi <i>et al.</i> , 2000; Bezanilla <i>et al.</i> , 2000; D'Souza <i>et al.</i> , 2001
Myo51p	V	Non-essential component of the contractile ring; required to align the mitotic spindle	Yes Through Co-IP	Win <i>et al.</i> , 2001; Mulvihill <i>et al.</i> , 2001; Gachet <i>et al.</i> , 2004
Myo52p	V	Involved in delivery of vesicles containing cell wall to the tips and maturation/cleavage of the septum	N/D** Through Co-IP	Win <i>et al.</i> , 2001; Mulvihill <i>et al.</i> , 2001; Gachet <i>et al.</i> , 2004; Mulvihill <i>et al.</i> , 2006

N/D**: Not detected

Co-IP: co-immunoprecipitation

binding site for the myosin essential light chain (ELC). When analyzed for its localization in relation to other cytokinesis proteins, Cdc4p was observed to localize to the contractile ring coincident with myosin as a broad equatorial band (Wu *et al.*, 2003).

Cdc4p has also been observed to interact with another myosin, Myo51p (Table 1.1). Myo51p is an unconventional type V myosin and non-essential component of the contractile ring (D'Souza *et al.*, 2001). Myo51p staining at the contractile ring is abolished in *cdc4*^{G107S} mutants (Win *et al.*, 2000). There are two other myosins found in *S. pombe* including Myo1p (a type I myosin) and Myo52p (a type V myosin). Co-immunoprecipitation experiments failed to detect an interaction between Cdc4p and Myo52p suggesting that Cdc4p may not interact with Myo52p (D'Souza *et al.*, 2001). Other methods to detect protein-protein interactions were not performed for Myo52p. It has not been shown if Cdc4p interacts with Myo1p. Table 1.1 summarizes the functions of the five different myosins in *S. pombe* and their interactions with Cdc4p.

1.1.3.6.1.1. The structure and regulation of muscle myosin

Myosin function has been characterized mainly in striated or smooth muscle cells, but studies of contraction have also been studied in the context of cytokinesis. The myosin heavy chain is ~140 nm long (Timson, 2003), including ~130 nm of the coiled-coil tail formed by the two heavy chains (Timson, 2003). The N-terminal region however, is globular and contains MgATPase activity, actin binding activity and the ELC and RLC binding sites (Timson, 2003). Studies have used mainly rabbit derived skeletal myosin (Timson, 2003). The neck region (also known as the lever arm domain) with its associated light chains plays both structural and regulatory roles (Timson,

2003). It is an 8.5 nm long α -helical heavy chain which consists of two IQ-motifs of the consensus sequence IQxxxRGxxxR which act as the attachment sites for the two myosin light chains (Rayment *et al.*, 1993a and b; Dominguez *et al.*, 1998). Muscle contraction occurs when filaments containing a molecular motor pull against another set of filaments which contain mainly actin (Timson, 2003). The source of energy for this directional movement is provided by the hydrolysis of MgATP catalyzed by myosin (Timson, 2003). The hydrolysis of MgATP is coupled to a series of conformational changes in myosin which result in a cycle of attachment, force development, release and reattachment (Iwamoto *et al.*, 2007; Eisenberg and Hill, 1985).

Owing to myosin's large and filamentous nature, biochemical characterization of this molecule is often difficult. As a result, most studies of myosin have been done with a soluble chymotryptic fragment called S1 (subfragment 1) which consists of one globular head domain containing the ELC binding site, but not the RLC binding site. Some studies have been performed on heavy meromyosin (HMM), a proteolytic fragment of myosin which contains two heads, a full set of light chains and part of the coiled-coil tail region.

In higher eukaryotes, multiple isoforms of the ELC exist. In skeletal muscle, there are two isoforms, A1 (also known as LC1) and A2 (also known as LC3) (Frank and Weeds, 1974). These isoforms are generated by differential mRNA splicing (Nabeshima *et al.*, 1984). They differ at their N-terminus where A1 has an extra 45 amino acids. In A1, the first 10 amino acids are rich in lysine residues and the remainder of the extension consists mainly of proline-alanine repeats. This N-terminal extension has been shown to mediate an additional interaction between the ELC and

actin (Labbe *et al.*, 1986). Studies on the A1 isoform of ELC are conflicting (Frank and Weeds, 1974). Studies with the S1 fragment of myosin suggest that A1 acts to make myosin bind to actin more tightly and hydrolyse MgATP more slowly (Weeds and Taylor, 1975; Wagner and Weeds, 1977). However, studies with HMM suggest that the A1 isoform of ELC may act to reduce myosin's affinity for actin (Stepkowski *et al.*, 1997).

The mechanism of contraction and the roles of myosin light chains varies greatly between organisms, but usually is triggered by increases in intracellular calcium. In some invertebrate myosins (i.e., scallop), contraction occurs through the direct binding of calcium to the ELC (Xie *et al.*, 1994). Binding of calcium to the ELC in scallop myosin requires that the RLC and myosin protein-protein interactions be present (Xie *et al.*, 1994). However, in mammalian cells, contraction (i.e., smooth muscle contraction) depends on the phosphorylation of the RLC on myosin by the myosin light chain kinase (MLCK) (Allen and Walsh, 1994). Likewise, muscle relaxation depends on the dephosphorylation of RLC on myosin through the myosin light chain phosphatase (MLCP). RLC phosphorylation by MLCK results from a signal transduction cascade involving PtdIns(4,5)P₂. Here, PtdIns(4,5)P₂ becomes cleaved by phospholipase C (PLC) generating diacylglycerol (DAG) and inositol trisphosphate (IP₃). IP₃ binds to the IP₃ receptor on the ER triggering the release of calcium. Alternatively, calcium may be transported into the cell from the extracellular space. Calcium then binds calmodulin and the calcium-calmodulin complex interacts with MLCK which in turn phosphorylates RLC on residues serine 19/threonine 18 (Allen

and Walsh, 1994). The major phosphorylation site is serine 19, which allows myosin II to interact with actin, initiating contraction.

RLC also gets phosphorylated through calcium independent mechanisms by the proteins integrin-linked kinase, Zip kinase, Rho-kinase, p21-activated kinase 3, p21-activated kinase γ , MAPKAP2, p90 ribosomal S6 kinase-2 and AIM-1 (Amano *et al.*, 1996; Komatsu *et al.*, 1996; Kureishi *et al.*, 1997; Chew *et al.*, 1998; Van Eyk *et al.*, 1998; Murata-Hori *et al.*, 1999; Suizu *et al.*, 2000; Murata-Hori *et al.*, 2000; Niino *et al.*, 2001). All these proteins phosphorylate RLC on serine 19. Overall, contraction in smooth muscle appears to be maintained through a balance of the activities of the MLCK and MLCP proteins in the cell.

In striated muscle (i.e., the muscles of the heart and skeleton) regulation occurs on the actin filament where troponin C binds to calcium (Farah and Reinach, 1995). This interaction leads to a series of conformational changes within the regulatory proteins of the thin filaments tropomyosin, and troponin T and I, which then leads to the interaction of the thick, myosin-containing filaments with the actin-containing filaments, force development and contraction. In striated muscle, phosphorylation of the RLC still occurs, however, the consequences of this phosphorylation are still poorly understood (Sweeney *et al.*, 1993; Ryder *et al.*, 2007).

These mechanisms of myosin dependent contraction differ from the unicellular systems of yeast and *Dictyostelium* which use alternative mechanisms to regulate contraction for cytokinesis (Matsumura, 2005). In *Dictyostelium*, myosin heavy chain phosphorylation, but not myosin light chain phosphorylation, plays an essential role in regulating cytokinesis (Ostrow *et al.*, 1994; De La Roche *et al.*, 2002). In fission yeast,

phosphorylation of RLC does not even seem to occur as myosin contraction is dependent on the binding of Rng3p to myosin II (Lord and Pollard, 2004). Furthermore, no orthologs of MLCK or MLCP are present in the genomes of yeast or *Dictyostelium* (Matsumura, 2005).

Overall, the ELC in higher eukaryotes has been observed to play more of a structural role in mediating myosin contraction whereas the RLC plays more of a regulatory role.

1.1.3.6.2. Cdc4p has multiple functions

Recent evidence, however, strongly suggests that Cdc4p is not just an ELC. First indications of this arose in the initial screen for *cdc* mutants identified by Nurse *et al.* (1976) in fission yeast. Nurse *et al.* (1976) noted that two alleles of *cdc4* (*cdc4-8* and *cdc4-31*) complemented in a diploid at the restrictive temperature. Although it was possible that these two alleles represented two different, but closely linked genes, this remained a genetic peculiarity of *cdc4*. The *cdc4-8* allele was shown to encode the *cdc4*^{G107S} mutation, and the *cdc4-31* allele was shown to encode the *cdc4*^{G19E} mutation (Desautels *et al.*, 2001). The interallelic complementation was later confirmed and the complementation of two additional diploid combinations of *cdc4* alleles at the restrictive temperature was shown (*cdc4*^{G82D}/*cdc4*^{F12L} and *cdc4*^{F12L}/*cdc4*^{R33K}) (Desautels *et al.*, 2001). The protein structures of three of the *cdc4* conditional mutants (G19E, G82D, and G107S) demonstrated that only localized perturbations in structure occurred at high temperatures suggesting that the loss-of-function phenotypes exhibited by these alleles was likely due to loss of protein-protein interactions rather than a global

disruption of the protein structure (Slupsky *et al.*, 2001). These observations suggest that the Cdc4p protein may dimerize or that it has more than one essential function.

Further studies showed that Cdc4p has multiple functions. The effects of mutations in *rlc1* are suppressed by the deletion of its binding site on Myo2p (IQ2) (Naqvi *et al.*, 2000). The *rlc1* gene is essential at 19°C, but not at 25°C or 35°C. For example, at 19°C, cells deleted for *rlc1* demonstrate cytokinesis defects and are not viable. However, *rlc1Δmyo2IQ2Δ* double-mutant cells are viable at 19°C (Naqvi *et al.*, 2000). This suggests that the only essential function of Rlc1p at 19°C is binding to Myo2p at IQ2. This is in contrast to the effects of mutations in *cdc4* that are not suppressed by the deletion of its binding sites on *myo2*. For example, *cdc4* temperature-sensitive strains (*cdc4*^{G107S} or *cdc4*^{R33K}) also mutated for Cdc4p binding sites on *myo2* (*myo2IQ1Δ* and *myo2IQ1ΔIQ2Δ*) did not grow at the non-permissive temperature. This suggests that Cdc4p has additional binding partners or functions that are essential for cytokinesis (D'Souza *et al.*, 2001).

Furthermore, experiments were performed in which cells deleted for *myp2* and at the same time deleted for both IQ domains on *myo2*, demonstrated that Cdc4p still localized to the actomyosin ring during cytokinesis (D'Souza *et al.*, 2001). Thus, there is another component of the contractile ring capable of interacting with Cdc4p besides the type II myosins Myo2p and Myp2p.

Actomyosin sliding filament assays were performed with crude Myo2p isolated from six temperature sensitive *cdc4* strains. Myosin motility was reduced in the *cdc4*^{R33K} strain at the non-permissive temperature, but not in the other five strains of *cdc4* conditional mutants, again providing evidence that *cdc4* may have multiple

functions aside from that of a myosin interactor (Lord and Pollard, 2004). Finally, the Cdc4p concentration in fission yeast cells is 10-fold higher than that of Myo2p or Rlc1p (Wu and Pollard, 2005). This is unusual since one would expect to have a 1:1:1 ratio of myosin, RLC and ELC molecules. If the sole function of Cdc4p is that of a myosin essential light chain, then one would expect to have equimolar ratios of Cdc4p and Myo2p. Altogether, these results provide evidence for Cdc4p interactions with other proteins aside from type II myosins in fission yeast.

Cdc4p has been shown to interact with at least one other IQ-domain containing protein that is not a myosin. Cdc4p functionally and physically interacts with Rng2p, the human IQGAP1 homologue in *S. pombe* (D'Souza *et al.*, 2001). IQGAP1 in mammalian cells integrates signaling pathways and co-ordinates several important cellular processes. It regulates actin cytoskeletal dynamics through Rac1 and Cdc42 and cell-cell adhesion through E-cadherin and β -catenin (Brown and Sacks, 2006). IQGAP1 does not have GTPase-activating protein (GAP) activity, but was named IQGAP because it has multiple IQ-motifs and has a Ras GAP homology domain. GAPs increase the intrinsic rate of GTP hydrolysis of GTPases thereby inactivating them. Contrary to its homology to GAPs, IQGAP1 binds to Cdc42 and Rac1, inhibiting their GTPase activity, stabilizing them in their active GTP-bound form. Calmodulin regulates IQGAP1 by affecting its protein binding activity in a Ca^{2+} -dependent and independent manner. IQGAP1 has also been shown to interact with itself and is regulated by phosphorylation (Brown and Sacks, 2006). *S. pombe* Rng2p contains multiple IQ-motifs (six), and based on its homology to IQGAP1, is thought to bind actin and calmodulin, and is likely a potential effector for the Rho family of GTPases like its

mammalian orthologue (Eng *et al.*, 1998). Rng2p is also a component of the contractile ring and of the SPB, and is required for assembly of the contractile ring (Eng *et al.*, 1998; Wu *et al.*, 2003). A synthetic lethal interaction was observed between the *cdc4*^{G107S} and *rng2-D5* alleles, and co-immunoprecipitation experiments demonstrated that they physically interact (D'Souza *et al.*, 2001).

The mutant strain *cdc4*^{G107S} was shown to be sensitive to FK506, which inhibits calcineurin function (Fujita *et al.*, 2002). The fission yeast genome has a single gene encoding the catalytic subunit of calcineurin, *ppb1*, which is essential for cell separation (Yoshida *et al.*, 1994). Calcineurin genetically interacts with Apm1p and Ypt3p described in this document. In *ppb1Δ* mutants, cytokinesis is delayed and the septum is highly disorganized, in contrast to contractile ring formation, which is normal. In the study by Fujita *et al.*, (2002), *cdc4*^{G107S} cells treated with FK506 at the permissive temperature were defective in contractile ring formation, leading to the suggestion that calcineurin may somehow functionally interact with Cdc4p.

Cdc4p also genetically interacts with a protein called Imp2p. Imp2p is homologous to the protein Cdc15p and mouse *proline, serine, threonine phosphatase interacting protein* (PSTPIP) (Demeter and Sazer, 1998). Both Cdc15p and PSTPIP are involved in contractile ring organization (Carnahan and Gould, 2003; Spencer *et al.*, 1997). Overexpression and gene deletion studies demonstrate that Imp2p is required for contractile ring assembly and septation. This comes from studies where cells deleted for *imp2* have aberrant incomplete septa and ring structures. Tetrad analysis of *imp2Δ cdc4*^{G107S} asci showed that cells with both the null allele of *imp2* and the *cdc4*^{G107S}

mutation did not grow at 25°C (Demeter and Sazer, 1998). This was a synthetic lethal interaction since both *imp2Δ* and *cdc4*^{G107S} single mutants are viable at 25°C.

Genetic screens to identify suppressors of *cdc4* revealed a genetic interaction between a heat shock protein homologous to *S. cerevisiae* HSP12, called *scf1* (suppressor of *cdc four*) (Jang *et al.*, 1996) and an allele of actin (*act1-48*) which affects actin patch formation, but does not perturb actin ring formation (McCollum *et al.*, 1999b). Additionally, a yeast two-hybrid screen was performed by Desautels and colleagues (2001) and two novel interactions were found. One was with a Vps27p-like protein, an endocytic protein sorting molecule, and the other interaction was with Pik1p, a putative phosphatidylinositol 4-kinase (Desautels *et al.*, 2001). Pik1p is similar in sequence to the *S. cerevisiae* Pik1p protein, a confirmed phosphatidylinositol 4-kinase (Flanagan *et al.*, 1993; Garcia-Bustos *et al.*, 1994). Furthermore, the Pik1p interaction with Cdc4p was abrogated with the *cdc4*^{G107S} sequence. However, Pik1p interacted with the other four *cdc4* temperature-sensitive alleles (*cdc4*^{F12L}, *cdc4*^{G19E}, *cdc4*^{R33K}, *cdc4*^{F79S} and *cdc4*^{G82D}) (Desautels *et al.*, 2001). All together, these results suggest that Cdc4p is more than just a myosin essential light chain.

1.2. Phosphatidylinositol (PtdIns) Metabolism

The putative interaction between Cdc4p and a PtdIns 4-kinase suggests a possible role for PtdIns metabolism in cytokinesis. Phosphoinositides are key signaling molecules required for signal transduction, vesicle trafficking, and cytoskeleton organization (Figure 1.8, Table 1.2) (Di Paolo and De Camilli, 2006). PtdIns and its

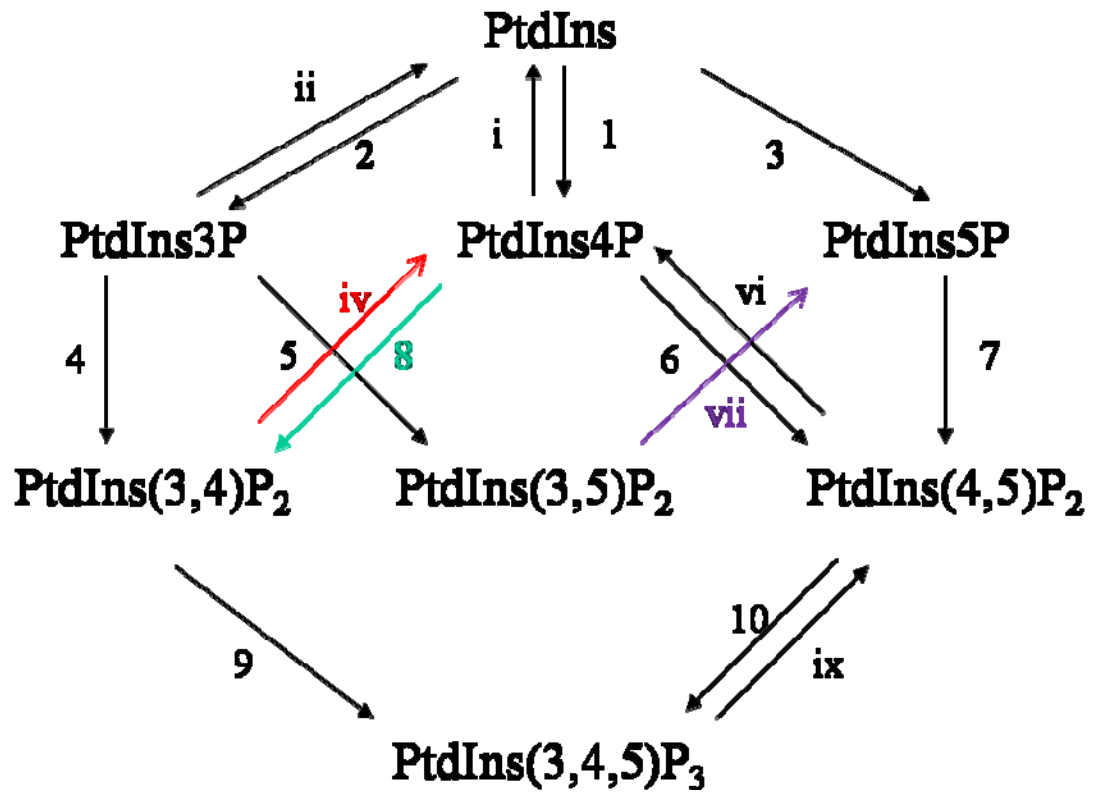


Figure 1.8: **PtdIns metabolism in yeast.**

PtdIns can be rapidly phosphorylated and dephosphorylated at various positions on the inositol head group (3', 4' and 5'). The spatially organized presence of PtdIns kinases, PtdInsP kinases and PtdIns phosphatases results in the discrete localization of PtdIns species located at precise locations in the cell. This figure is a summary of the different PtdIns species found in yeast cells and their pathways of formation.

Table 1.2.

A summary of most of the known PtdIns kinases and phosphatases in *S. cerevisiae* and

S. pombe.

Phosphoinositide kinases

	Pathway	<i>S. cerevisiae</i>	<i>S. pombe</i>
PtdIns 3-kinase	2, 10	Vps34p	Pik3p
		Tor1p	Tor1p
		Tor2p	Tor2p
PtdIns 4-kinase	1	Lsb6p	SPAC343.19/SPAC824.01
	1	Pik1p	Pik1p
	1	Stt4p	SPBC577.06
PtdIns 5-kinase	3?, 5	Fab1p	Fab1p
PtdIns4P 5-kinase	6, 9	Mss4p	Its3p

Phosphoinositide phosphatases

	Pathway	<i>S. cerevisiae</i>	<i>S. pombe</i>
3-phosphatases	iv, ix	Tep1p	Ptn1p
	ii, vii?	Ymr1p	SPAC19A8.03
4-phosphatases	i	Sac1p	SPAC3C7.01c
	i	Inp52p, Inp53p	Syj1p, Syj2p
5-phosphatases	vi	Inp52p, Inp53p	Syj1p, Syj2p
	vi	Inp54p, Inp51p	Syj1p, Syj2p

phosphorylated derivatives (PtdInsPs), are found in cell membranes and consist of approximately 10% of total cellular phospholipids (De Matteis and Godi, 2004). PtdIns can undergo rapid phosphorylations and dephosphorylations by PtdIns kinases and phosphatases which add or remove phosphates at positions 3', 4' and 5' of the inositol head group (Figure 1.8, Table 1.2). Discrete localization of PtdIns metabolizing enzymes can be found within different compartments of the cell. As a result, the plasma membrane contains mainly PtdIns(4,5)P₂, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. The Golgi contains mainly PtdIns4P and endosomes contain mainly PtdIns3P and PtdIns(3,5)P₂. These lipids contribute to endocytic, exocytic and degradative processes in the cell, respectively (Fruman *et al.*, 1998; De Matteis and Godi, 2004).

There are seven PtdInsP metabolites derived from PtdIns (Figure 1.8). There are three monophosphoinositides; PtdIns 3-phosphate (PtdIns3P), PtdIns 4-phosphate (PtdIns4P) and PtdIns 5-phosphate (PtdIns5P); three bisphosphoinositides, PtdIns 3,4-bisphosphate (PtdIns(3,4)P₂), PtdIns 3,5-bisphosphate (PtdIns(3,5)P₂), and PtdIns 4,5-bisphosphate (PtdIns(4,5)P₂); and one trisphosphoinositide, PtdIns 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) (Fruman *et al.*, 1998; De Matteis and Godi, 2004). The lipid kinases involved in producing these derivatives are PtdIns 3-kinases, PtdIns 4-kinases and PtdIns-phosphate (PtdInsP) kinases (Table 1.2). PtdIns 3-kinases phosphorylate the 3rd position of the inositol head group, producing PtdIns3P, PtdIns(3,4)P₂ or PtdIns(3,4,5)P₃. PtdIns 4-kinases phosphorylate the 4th position of the inositol head group, producing only PtdIns4P. PtdInsP kinases phosphorylate the 4th or 5th position of phosphorylated phosphoinositides, producing only PtdIns(4,5)P₂. In addition, there is a PtdIns3P 5-kinase, producing PtdIns(3,5)P₂. PtdIns 3-kinases,

PtdIns 4-kinases or PtdInsP kinases are further classified based on their biochemical features and sequence similarities (Fruman *et al.*, 1998; Lindmo and Stenmark, 2006; Balla and Balla, 2006).

1.2.1. PtdIns are involved in Golgi assembly and function

Phosphoinositides are important molecules required for cell signaling, membrane trafficking and cytoskeletal rearrangements. PtdIns(4,5)P₂ is a key player in cell signaling whereby PtdIns(4,5)P₂ is cleaved by phospholipase C producing diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP₃). IP₃ binds to receptors on the ER causing the translocation of Ca²⁺ into the cytoplasm causing a range of cellular responses such as cell differentiation, muscle contraction and cell proliferation (Lee and Rhee, 1995).

PtdIns also play critical roles in membrane trafficking. One good example is the role of PtdIns4P at the *trans*-Golgi. The *trans*-Golgi network is a major site of sorting and packaging of proteins and lipids to be delivered to distinct subcellular locations. PH domains that are specific for PtdIns4P *in vitro* localize predominantly to the Golgi indicating that the main PtdIns species at the Golgi is PtdIns4P. However, PtdIns(4,5)P₂ also plays a role in membrane trafficking from the Golgi, even though it is not as abundantly localized as its precursor PtdIns4P, as will be described.

The Golgi apparatus is a dynamic organelle made up of stacks of flat cisternae. In animal cells the number of cisternae in a single stack varies, but is usually ~7 (Nakano, 2004). Mammalian Golgi is single copy and clustered around the perinuclear region. Its structure is dependent on microtubules and indeed, a microtubule organizing center

is located perinuclearly as well (Nakano, 2004). Plant, insect and yeast cells do not have a single copy clustered Golgi, but have multiple Golgi scattered throughout the cytoplasm. In *S. cerevisiae*, the Golgi do not appear to be stacked which is different from some other Golgi, like that of *S. pombe* or *Pichia pastoris*, which appear to be stacked (Nakano, 2004). In *S. cerevisiae*, Golgi biogenesis appears to occur by two mechanisms: *de novo* synthesis at ER exit sites, and an actin-myosin dependent partitioning system, where the partitioned Golgi are targeted to the bud site during cell division (Rossanese *et al.*, 1999; Rossanese *et al.*, 2001; Bevis *et al.*, 2002; Lowe and Barr, 2007).

There are two main PtdIns 4-kinases at the Golgi, PtdIns4KII α and PtdIns4KIII β . PtdIns4KII α , unlike PtdIns4KIII β , is recruited to the Golgi in an Arf1-independent manner since the association of PtdIns4KII α from the Golgi does not change with the addition of BFA, a known Arf1 inhibitor (Wang *et al.*, 2003). In cells treated with siRNA against PtdIns4KII α , a large decrease in the formation of PtdIns4P at the Golgi was observed through the use of anti-PtdIns4P antibodies and a GFP tagged PH domain of oxysterol binding protein (OSBP). The knock-down of PtdIns4KII α also caused the dissociation of the γ -adaptin subunit of AP-1 from the Golgi. γ -adaptin is an endosomal and trafficking adaptor of the adaptor protein complex family. This was an effect specific to PtdIns4KII α and not PtdIns4KIII β or PtdIns4KII β since knock-down of these enzymes did not cause the γ -adaptin subunit to dissociate from the Golgi (Wang *et al.*, 2003). The association of clathrin with the Golgi was also greatly reduced, consistent with the idea that AP-1 acts as an accessory protein involved in the formation of clathrin-coated vesicles. AP-1 binds to PtdIns4P *in vitro*, and the addition

of PtdIns4P in cells knocked-down for PtdIns4KII α rescued the recruitment of AP-1 to the Golgi (Wang *et al.*, 2003). A model for studying pathways of export of ER proteins to the plasma membrane is the temperature-sensitive ts045 variant of VSVG (vesicular stomatitis virus G) which contains a single luminal amino acid change that leads to misfolding and retention in the ER at 40°C. Upon shifting to 32°C, misfolding is reversed and VSVG is exported out of the ER for delivery to the plasma membrane (Bergmann, 1989; Presley *et al.*, 1997). VSVG delivery out of the ER to the plasma membrane can be monitored through the use of anti-VSVG antibodies. Cells knocked-down for PtdIns4KII α displayed defects in the secretion of VSVG. The addition of PtdIns4P and PtdIns(4,5)P₂ rescued this defect in secretion (Wang *et al.*, 2003). This implicates both PtdIns4P and PtdIns(4,5)P₂ in the process of secretion. It is interesting to note that AP-1 also becomes recruited to the Golgi in an Arf1-dependent manner (Zhu *et al.*, 1998; Zhu *et al.*, 1999; Bonifacino and Lippincott-Schwartz, 2003).

The knock-down of PtdIns4KII α also decreased the association of GGA proteins (Golgi localized, γ -adaptin ear homology domain, Arf binding) with the Golgi (Wang *et al.*, 2007). They are called γ -adaptin ear homology domain proteins because they have a conserved γ -adaptin domain similar to that of the γ -adaptin subunit of AP-1. There are 3 human GGA proteins and all three were displaced in response to PtdIns4P depletion (Wang *et al.*, 2007). GGA1 and GGA2 GAT domains bind to PtdIns4P (Boman, 2001; Wang *et al.*, 2007). GGA proteins interact with Arf1-GTP and become recruited to the Golgi in an Arf1-dependent manner (Puertollano *et al.*, 2001; Takatsu *et al.*, 2002). PtdIns4P liposomes also promoted GGA binding to ubiquitinated-agarose (Wang *et al.*, 2007). Ubiquitin acts as a sorting signal in membrane trafficking (van der

Goot and Gruenberg, 2006). Thus, the dual requirement for PtdIns4P and Arf1-GTP allows GGA proteins to behave as sensitive coincidence detectors that associate with the *trans*-Golgi network in a spatially and temporally appropriate manner. Coincidence detection refers to the recruitment of proteins through several relatively low affinity interactions. In this case a protein-protein interaction and a lipid-protein interaction.

The second Golgi localized PtdIns 4-kinase, PtdIns4KIII β , also generates a pool of PtdIns4P required for the recruitment of *trans*-Golgi localized proteins. Arf1-GTP interacts with PtdIns4KIII β recruiting it to the *trans*-Golgi along with an as of yet unidentified PtdIns4P 5-kinase (Godi *et al.*, 1999). This recruitment causes an increase in the production of PtdIns4P and PtdIns(4,5)P₂ at the Golgi. An example of two proteins that are recruited by the PtdIns4KIII β pool of PtdIns4P is the four-phosphate adapter proteins FAPP1 and FAPP2 (Godi *et al.*, 2004). Both FAPPs became dissociated from the *trans*-Golgi when the kinase-dead PtdIns4KIII β D656A allele was expressed. Expression of this PtdIns4KIII β allele also causes changes in Golgi structure (Godi *et al.*, 1999). The PH domains of the FAPPs were shown to be specific for PtdIns4P and were also involved in binding with Arf1-GTP, stabilizing its activation state by interfering with its interaction with Arf1-GAP1 (GTPase activating protein) at Golgi membranes (Godi *et al.*, 2004). GGA proteins have been observed to have similar Arf1 stabilizing mechanisms, through their GAT domains (Boman, 2001). In cells knocked-down for FAPP1 and FAPP2, VSVG transport to the plasma membrane was inhibited suggesting a role for FAPP1 and FAPP2 in secretion. It is currently unknown if these FAPP proteins interact with the vesicle forming machinery at the Golgi.

In *S. cerevisiae*, the loss-of-function of *pik1* (*pik1-83*), the PtdIns4KIII β homologue, causes the PH domains of OSBP (Osh1p) and FAPP1 to dissociate from the Golgi (Levine and Munro, 2002). The Golgi localization of the PH domain however, was unaffected in cells with a loss-of-function mutant of *stt4* (*stt4-4*), *vps34* (*vps34 Δ*) and *mss4* (*mss4-2*), a PtdIns 4-kinase, PtdIns 3-kinase and PtdIns4P 5-kinase, respectively. A mutation in the PH domain of OSBP (R107E, R108E), which removed its binding to PtdIns4P, did not cause dissociation of the PH domain from the Golgi suggesting that the presence of another Golgi dependent factor was exerting effects on the localization of the PH domain (Levine and Munro, 2002). The PH domain mutant did delocalize from the Golgi in an *arf1-11* background suggesting that recruitment of the PH domain to the Golgi requires both Arf1p and PtdIns4P. Overexpression of the PH domain was dominant-negative and caused disruptions in Golgi structure analogous to cells with the *arf1-11* loss-of-function mutant (Levine and Munro, 2002). In mammalian cells, the PH domain of OSBP was shown to pull-down Arf1 in immunoprecipitation experiments (Godi *et al.*, 2004).

EpsinR is another protein required for membrane trafficking at the *trans*-Golgi. It binds PtdIns4P, clathrin and the γ -appendage of AP-1 and GGA proteins (Mills *et al.*, 2003; Hirst *et al.*, 2003; Legendre-Guillemain *et al.*, 2004). The ENTH (epsin N-terminal homology) domain of EpsinR binds PtdIns4P and is conserved with the Epsin1 ENTH domain known to be involved in clathrin mediated endocytosis at the plasma membrane. The Epsin1 ENTH domain structure has a “helix 0” where upon binding to PtdIns(4,5)P₂, the helix will unfold. The helix is known to then insert itself between

lipid head groups aiding in membrane curvature during clathrin cage formation. An analogous mechanism is proposed for EpsinR (Mills *et al.*, 2003).

As suggested by the data above, PtdIns(4,5)P₂ also is required for membrane trafficking from the *trans*-Golgi. Two proteins that are localized to the Golgi and bind to PtdIns(4,5)P₂ are spectrin and dynamin. Spectrin is localized to Golgi membranes in response to Arf1 activation and through binding to PtdIns(4,5)P₂ at the Golgi (Godi *et al.*, 1998). Dynamin is a large GTPase that oligomerizes at the bud neck of clathrin-coated vesicles and is involved in fission of the vesicle from the *trans*-Golgi network. The affinity of dynamin's PH domain to PtdIns(4,5)P₂ is low (Praefcke and McMahon, 2004). However, once bound to the phosphoinositide, oligomerization of the protein occurs and a strong interaction with the membrane is achieved. It is interesting to note that mutations in the PH domain of dynamin have dominant-negative effects. Fission is attained through the hydrolysis of GTP brought upon by the oligomerization of dynamin (Praefcke and McMahon, 2004). Thus, PtdIns4P and its derivative PtdIns(4,5)P₂ is critical for the assembly of many accessory proteins involved in secretion at the *trans*-Golgi network. Moreover, PtdIns4P appears to act as a coincidence sensor facilitating additional interactions with other proteins involved in budding from the *trans*-Golgi network.

1.2.2. PtdIns, contractile ring formation and cytokinesis

A variety of actin-binding proteins have also been reported to contain binding sites for phosphoinositides (Sechi and Wehland, 2000; Yin and Janmey, 2003).

PtdIns(4,5)P₂ in particular is important for polymerization and depolymerization events

of the actin cytoskeleton. Increasing the concentration of PtdIns(4,5)P₂ stimulates actin polymerization, whereas low levels trigger disassembly (Janmey and Lindberg, 2004). Proteins that sever or depolymerize actin filaments such as gelsolin, villin, cofilin and profilin are inactivated by PtdIns(4,5)P₂ *in vitro*. Proteins that link actin filaments to each other or to the membrane, such as vinculin, talin, ezrin and α -actinin are activated by PtdIns(4,5)P₂ *in vitro*.

PtdIns and PtdInsPs are involved in cytokinesis. Several studies with PtdIns metabolism inhibitors have shown that PtdIns are involved in cytokinesis. First indications were through the use of lithium (Li⁺) in sea urchin zygotes (Forer and Sillers, 1987; Becchetti and Whitaker, 1997). Here, ingression of the cleavage furrow occurred, but later regressed producing large multinucleate cells. Li⁺ blocks the conversion of IP₃ to inositol by inhibiting inositol monophosphatase and inositol polyphosphate-1-phosphatase (Naccarato *et al.*, 1974; Parthasarathy and Eisenberg, 1986; Rana and Hokin, 1990 for reviews). Lithium also inhibits cytokinesis in the crane fly likely by preventing the formation of new PtdIns (Saul *et al.*, 2004). This suggests that continued production of PtdIns, likely required for the production of PtdIns4P and PtdIns(4,5)P₂, is required for cytokinesis in insect cells. Lithium, and wortmannin (which blocks the activity of PtdIns 3-kinases) both slowed furrow ingression (Vemuri *et al.*, 1996). Recently, the PtdIns pathway has been observed to be important for cytokinesis in crane fly and *Drosophila* spermatocytes (Saul *et al.*, 2004; Wong *et al.*, 2005). The phospholipase C inhibitor U7 demonstrated cytokinesis defects in crane fly and *Drosophila* male germline cells indicating that the hydrolysis of PtdIns(4,5)P₂ and perhaps DAG, IP₃ and calcium were continuously required for maintaining the cleavage

furrow and continuing ingression (Saul *et al.*, 2004). Injection of antibodies against PtdIns(4,5)P₂ and addition of neomycin, which depletes PtdIns(4,5)P₂ pools, also results in cytokinesis defects (Han *et al.*, 1992; Zhang *et al.*, 2000).

Other PtdIns metabolic enzymes have also been shown to be involved in cytokinesis, and have been found at the division site, namely Its3p in fission yeast (Zhang *et al.*, 2000). Its3p is a PtdIns4P 5-kinase which localizes to the division plane, along with its product PtdIns(4,5)P₂. Its3p was initially discovered in a screen for mutants sensitive to the calcineurin inhibitor FK506 and high temperature (Zhang *et al.*, 2000). *its3* is an essential gene and homologous to the budding yeast Mss4p PtdIns4P 5-kinase. Calcineurin, as described previously, is required for cell separation in fission yeast (Section 1.1.3.6.2 and Section 1.1.3.5.2). The growth of *its3-1* cells is dependent on calcineurin suggesting that these two proteins share essential overlapping functions (Zhang *et al.*, 2000). The *its3-1* allele demonstrated decreased PtdIns(4,5)P₂ and increased PtdIns4P levels in the cell suggesting that Its3p is indeed a PtdIns4P 5-kinase. Mutants of Its3p demonstrate disrupted actin patch formation, but normal actin ring formation suggesting that it is not involved in contractile ring formation. The *its3-1* mutant showed a doubling in the number of cells with septa at the permissive temperature, and a much more dramatic increase in the number of cells with septa (24%-50%) at the non-permissive temperature indicating a cell separation defect (Zhang *et al.*, 2000). Its3p, like its product PtdIns(4,5)P₂, is localized to the medial region of the cell suggesting that Its3p acts in cytokinesis by directly generating PtdIns(4,5)P₂ at the site of cell division. Similar results were obtained with studies of the mammalian PtdIns4P 5-kinase homologue in Chinese hamster ovary fibroblasts (Emoto *et al.*,

2005). Here, the PtdIns4P 5-kinase and its product PtdIns(4,5)P₂ were also observed to localize to the cleavage furrow and shown to be involved in the completion of cytokinesis.

PTEN is a PtdIns(3,4,5)P₃ phosphatase that has been implicated in cytokinesis in *D. discoideum* (Janetopoulos *et al.*, 2005). The formation of PtdIns(3,4,5)P₃ was monitored through the use of a PH domain specific for PtdIns(3,4,5)P₃ (PH_{Crac}) (Postma *et al.*, 2004). During cytokinesis, PtdIns(3,4,5)P₃ forms at the poles of the cells and is absent from the medial region. Consistent with this, the PtdIns 3-kinase PI3K2-GFP was observed at F-actin based ruffles at the poles and PTEN was observed at the medial region. Disruption of both PtdIns 3-kinases (PI3K1 and PI3K2) and PTEN, resulting in unmodulated PtdIns(3,4,5)P₃, generated cells that were large and multinucleate. The addition of a PTEN mutant which lacked its N-terminal PtdIns(4,5)P₂ binding motif failed to interact with the membrane and failed to rescue the defects of *pten*⁻ cells. Since PtdIns(3,4,5)P₃ promotes the formation of actin based projections, it was hypothesized that the absence of PtdIns(3,4,5)P₃ at the furrow inhibits actin based projections in this region allowing actomyosin contractions to occur. Indeed, a PTEN homologue has been identified in fission yeast, Ptn1p, which localizes to the septum (Mitra *et al.*, 2004).

Studies of Fwd in *Drosophila*, a PtdIns 4-kinase, and Pik1p, its orthologue in budding yeast, demonstrate cytokinesis defects when mutated (Garcia-Bustos *et al.*, 1996; Brill *et al.*, 2000). Both Pik1p and Fwd are enriched on Golgi structures suggesting a role for secretion in cytokinesis (Walch-Solimena and Novick, 1999; Brill *et al.*, 2000). In Fwd mutants, the contractile ring constricts, but the cleavage furrow is

unstable and later regresses. Thus, secretion is involved in maintaining the contractile ring as well as maintaining the stability of the cleavage furrow (Brill *et al.*, 2000). PITPs are highly conserved proteins that bind phosphatidylinositol or phosphatidylcholine monomers, facilitating their transfer from one membrane compartment to another. Mutations in the *Drosophila* class I PITP Giotto results in a failure of actomyosin ring constriction and affects the distribution of Golgi-derived vesicles in dividing spermatocytes (Giansanti *et al.*, 2006). Thus, Giotto is required for normal insect cell cytokinesis. A mammalian class II PITP, Nir2, homologous to *Drosophila* RdgB, has also been shown to be required for cytokinesis. Both Nir2 and Giotto are enriched on Golgi structures (Litvak *et al.*, 2002; Lev, 2004; Giansanti *et al.*, 2006).

Overall, these results suggest that PtdIns are required for septation in *S. pombe* and late cytokinetic events in insect and mammalian cells. Studies in *D. discoideum* and *Drosophila* suggest that PtdIns metabolites may be involved in actin based activities, such as contractile ring contraction during cytokinesis that have not been shown in mammalian or yeast cells.

1.2.3. PtdIns 4-kinases

1.2.3.1. Biochemical features and classification

The PtdIns 4-kinases have been classified as either type II or type III based on their biochemical behavior and sequence similarity (Table 1.3) (Balla, 1998; Gehrman and Heilmeyer, 1998; Heilmeyer *et al.*, 2003, for reviews). The type II enzyme is inhibited by ATP about 20-fold more strongly than the type III enzyme. Furthermore,

Table 1.3.

PtdIns 4-kinases in budding yeast and mammalian cells, and their properties

Features	PtdIns4KIIα	PtdIns4KIIβ	PtdIns4KIIIα	PtdIns4KIIIβ
Other names	PI4KII; PI4K55; 56 kDa like PtdIns 4-kinase	PI4KII; PI4K55; 56 kDa like PtdIns 4-kinase	Pik4ca; PI4K230; PtdIns 4-kinase α	Pik4cb; PI4K92; PtdIns 4-kinase β
<i>S. cerevisiae</i> homolog	Lsb6p (Pik2p)	Lsb6p (Pik2p)	Stt4p	Pik1p
Apparent molecular weight	55-56 kDa	55-56 kDa	210 kDa	110 kDa
Calculated molecular weight	54 kDa	55 kDa	230 kDa	92 kDa
Wortmannin	Insensitive	Insensitive	IC ₅₀ 50-300 nM	IC ₅₀ 50-300 nM
LY-294002	Insensitive	Insensitive	IC ₅₀ 50-100 μ M	IC ₅₀ 100 μ M
Phenylarsine oxide	IC ₅₀ > 100 μ M	IC ₅₀ > 100 μ M	IC ₅₀ 1-5 μ M	IC ₅₀ ~ 30 μ M
Ca ²⁺	Inhibits	Inhibits	No direct effect	No direct effect
K _i (adenosine)	10-70 μ M	10-70 μ M	Millimolar	Millimolar

Triton X-100	Activates	Activates	Activates	Activates
K _m (ATP)	10-50 μM	10-50 μM	~ 700 μM	~ 400 μM
K _m (PtdIns)	~20-60 μM	~20-60 μM	~100 μM	~100 μM

K_m values for the PtdIns and ATP/Mg²⁺ substrates are 3-7-fold lower for the type II enzyme than for the type III enzyme. The type II enzyme is also sensitive to calcium, while having no effect on the type III enzyme. The type II enzyme however, is insensitive to the PtdIns 3-kinase inhibitors wortmannin and LY 294002, while the type III enzymes are only partially sensitive. The type II enzymes are also associated with membranes and require detergents for solubilization while the two type III enzymes are partially soluble to soluble. The various biochemical features of the type II and III PtdIns 4-kinase enzymes are summarized in Table 1.3.

1.2.3.2. PtdIns 4-kinases in *S. cerevisiae*

There are 3 PtdIns 4-kinases in *S. cerevisiae*: Lsb6p, Pik1p and Stt4p. All three play biologically important roles in the cell and will be discussed below.

1.2.3.2.1. Lsb6p

Lsb6p is homologous to mammalian PtdIns 4-kinase II α and was named Lsb6p because of its interaction with Las17p (Las17p-binding) (Madania *et al.*, 1999). Las17p is the orthologue of the human *Wiskott-Aldrich syndrome protein* (WASp), a promoter of actin filament polymerization. Lsb6p is a lipid kinase because its over expression increased PtdIns4P levels, and this elevation did not require Pik1p or Stt4p (Han *et al.*, 2002). Lsb6p is non-essential which is different than the other two essential PtdIns 4-kinases, Stt4p and Pik1p (Han *et al.*, 2002; Shelton *et al.*, 2003). It behaves like an integral membrane protein and localizes to the plasma membrane and vacuolar membrane (Han *et al.*, 2002; Shelton *et al.*, 2003). Mammalian type II PtdIns 4-kinases

are palmitoylated *in vivo* and protein palmitoylation software predicts that Lsb6p may be palmitoylated *in vivo* as well (Zhou *et al.*, 2006). This provides a mechanism for membrane targeting different than the other two PtdIns 4-kinases which require accessory proteins to be targeted to the membrane (Herman *et al.*, 1991a; Herman *et al.*, 1991b; Stack *et al.*, 1993; Hendricks *et al.*, 1999; Ames *et al.*, 2000; Audhya and Emr, 2002). Overexpression of Lsb6p partially rescues the inviability of *stt4* null mutants suggesting that PtdIns 4-kinase activity at the plasma membrane may be redundant (Audhya and Emr, 2002). Also, overexpression of *LSB6* causes no obvious phenotype (Han *et al.*, 2002; Shelton *et al.*, 2003).

Lsb6p localizes to the vacuolar membrane and studies suggest that PtdIns(4,5)P₂ may be involved in homotypic vacuole fusion. *In vitro* assays with α -PtdIns(4,5)P₂ antibodies or the addition of phospholipase C, which disrupt PtdIns(4,5)P₂ levels, disrupt vacuole fusion (Flick and Thorner, 1993; Mayer *et al.*, 2000). Moreover, the readdition of PtdIns(4,5)P₂ reverses this affect (Flick and Thorner, 1993; Mayer *et al.*, 2000). If true, then Lsb6p may supply the PtdIns4P necessary to generate PtdIns(4,5)P₂ required for homotypic vacuole fusion.

Lsb6p may also play a role in endosome motility with its association with Las17p (Chang *et al.*, 2005). The speed of endosome movement is significantly altered in *lsb6Δ* mutants compared to wild-type. Interestingly, *lsb6Δ* cells complemented with lipid kinase dead forms of *lsb6* or with the kinase domain deleted, have normal endosome speeds suggesting that endosome movement is independent of its catalytic activity. Lsb6p interacts with the N-terminal region of Las17p which then activates the

Arp2/3 complex. Stt4p, Pik1p and Mss4p are not required for endosome motility based on studies of loss-of-function mutants of these proteins.

1.2.3.2.2. Pik1p

Stt4p and Pik1p are essential type III PtdIns 4-kinases. The absence of either *STT4* or *PIK1* cannot be compensated by overexpressing the other indicating that each lipid kinase regulates a discrete pool of PtdIns4P required for different essential processes (Flanagan *et al.*, 1993; Cutler *et al.*, 1997; Audhya *et al.*, 2000). Both Stt4p and Pik1p generate the bulk of PtdIns4P in *S. cerevisiae*. Cells carrying a temperature-sensitive *stt4* mutation (*stt4-4*) display a 40% reduction in PtdIns4P and a 60% reduction in PtdIns(4,5)P₂. Whereas cells with a temperature-sensitive *pik1* mutant (*pik1-83*) display a 45% reduction in PtdIns4P and a 40% reduction in PtdIns(4,5)P₂. Cells carrying both mutations display a reduction of both phosphoinositides by >90% (Audhya *et al.*, 2000). Stt4p is 50% identical to PtdIns4KIII α and Pik1p is 42% identical to PtdIns4KIII β (Meyers and Cantley, 1997). Both Pik1p and Stt4p contain a lipid kinase unique (LKU) domain similar to that found in the PtdIns 3-kinase, Vps34p (Nakagawa *et al.*, 1996). In Pik1p, there is a novel homology domain (NHD), also known as the BSM domain (β signature motif), a hallmark of type III β PtdIns 4-kinases (Brill *et al.*, 2000; Strahl and Thorner, 2007). Stt4p has a PH domain in between the LKU domain and the catalytic domain, a diagnostic feature of type III α PtdIns 4-kinases (Gehrmann and Heilmayer, 1998; Stevenson *et al.*, 1998; Xue *et al.*, 1999).

The gene encoding the Frq1p protein is essential and binds tightly with Pik1p. It is a small Ca²⁺-binding protein and is required for the activation of Pik1p lipid kinase

activity (Hendricks *et al.*, 1999). *FRQ1*, named after frequenin-like protein in *S. cerevisiae*, like *cdc4*, is an EF-hand protein. Both are similar in sequence to each other (47.8% similarity; 23.9% identity). Studies of the mammalian orthologue of this protein indicate that this interaction and its effect on PtdIns 4-kinase activity are conserved (Bourne *et al.*, 2001; Zhao *et al.*, 2001; Kapp-Barnea *et al.*, 2003; Haynes *et al.*, 2005). Frq1p is orthologous to *Drosophila* frequenin and mammalian neuronal calcium sensor 1 (NCS-1). Expression of frog frequenin (Hendricks *et al.*, 1999) or human NCS-1 (Strahl *et al.*, 2003) rescues the lethality of *frq1Δ* cells, whereas other members of the same family, such as bovine recoverin or human kChIP2 fail to complement the *frq1* deletion mutant. *frq1Δ* cells were viable if *PIK1* was overexpressed suggesting that Pik1p is the sole essential target of Frq1p (Hendricks *et al.*, 1999). Furthermore, two *PIK1* conditional alleles carrying mutations in the LKU domain (*pik1-11* and *pik1-24*) were rescued by *FRQ1* ectopic expression at the restrictive temperature suggesting a functional interaction between both proteins (Hendricks *et al.*, 1999). Frq1p N-myristoylation is required for its function because non-myristoylated Frq1p (G2A) failed to fully rescue the conditional lethality of the *pik1-11* allele in a *frq1Δ* background (Hendricks *et al.*, 1999). Evidence for a direct physical interaction was demonstrated by co-immunoprecipitation experiments and the interaction was not dependent on myristoylation. Later structural studies demonstrated that Frq1p does not interact with Pik1p via the LKU domain, but through residues slightly C-terminal to those corresponding to the LKU domain and is bipartite (residues 129-136 and 157-169) (Huttner *et al.*, 2003). Pik1p and Frq1p form a 1:1 complex in a Ca^{2+} -dependent manner. Frq1p was first thought to be a calcium-myristoyl switch

where in the calcium-free state, it has the myristoyl-group tucked away in a hydrophobic cavity. In the calcium-bound state, major conformational changes occur such as disruption of the hydrophobic groove and release of the myristoyl group (Meyer and York, 1999). The protein structure of Frq1p was later solved and it was determined that Frq1p is not a calcium-myristoyl switch since no major conformational changes were observed in the presence of calcium and the myristoyl group is constitutively exposed (Ames *et al.*, 2000). The N-myristoyl group of Frq1p is thought to target Pik1p to the Golgi membrane since Pik1p lacks any obvious membrane or lipid binding motifs (Hendricks *et al.*, 1999; Ames *et al.*, 2000). This was confirmed by a *pik1* mutant (*pik1 Δ 152-191*) that could not bind Frq1p and localized diffusely throughout the cytoplasm, whereas wild-type Pik1p localizes to cytoplasmic puncta confirmed to be Golgi through co-localization experiments with Sec7p and Chs5p (Franzusoff *et al.*, 1991; Santos and Snyder, 1997; Walch-Solimena and Novick, 1999; Strahl *et al.*, 2005). Frq1p also localizes to the Golgi since it co-localized with Pik1p and Sec7p.

Co-localization of Pik1p with the *trans*-Golgi was also observed with the Chs5p and Kex2p *trans*-Golgi markers and corroborated with sucrose density gradient analysis (Walch-Solimena and Novick, 1999). In addition, indirect immunofluorescence indicates that Pik1p localizes to both the cytoplasm and nucleus in agreement with previous biochemical studies (Walch-Solimena and Novick, 1999). Pik1p was shown to undergo nucleocytoplasmic shuttling as demonstrated by mutants which lack the exportin and importin proteins Msn5p and Kap95p, respectively (Strahl *et al.*, 2005). In mutants lacking the exportin, Msn5p, there was a dramatic accumulation of Pik1p in the nucleus, and in mutants lacking the β -importin, Kap95p,

there was little to no Pik1p in the nucleus. Frq1p, or its binding to Pik1p, is not required for Pik1p nucleocytoplasmic shuttling, and the catalytic domain of Pik1p (residues 793-1066) is not required for its binding to Kap95p (Strahl *et al.*, 2005).

Catalytically active Pik1p in the nucleus is essential for yeast cell viability. The C-terminal CCAAX motif of yeast Ras2p is both S-palmitoylated and S-prenylated *in vivo* and acts as a constitutive membrane anchor (Hancock, 2003). The five C-terminal residues of Pik1p (QGIYS) were replaced with the five C-terminal residues of Ras2p (CCIIS), designated CCAAX. Pik1p-CCAAX constitutively localizes to the Golgi apparatus and displays wild-type levels of catalytic activity. The expression of the Pik1p-CCAAX construct does not rescue the viability of *pik1Δ* cells suggesting that Pik1p has other essential functions aside from that at the Golgi (Strahl *et al.*, 2005). On the other hand, *pik1(Δ10-192)* which lacks its nuclear export signal and Frq1p-binding site, retains its catalytic activity and accumulates in the nucleus. Expression of this allele in *pik1Δ* cells also fails to rescue growth suggesting that Pik1p function at the Golgi is essential for cell viability. Co-expression of both these constructs in *pik1Δ* cells restores growth consistent with its roles at the Golgi and nucleus being essential for Pik1p function. Experiments with catalytically inactive derivatives of these constructs show that Pik1p must be catalytically active to maintain function at both the Golgi and the nucleus (Strahl *et al.*, 2005).

As described earlier in this section through studies in mammalian cell systems, PtdIns4P production at the Golgi is important for secretion. The same holds true in yeast (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). Pik1p was first identified as being involved in secretion when it was found that *sec14-3*

temperature-sensitive mutants exhibited a drop in PtdIns4P levels at the restrictive temperature (Hama *et al.*, 1999). *SEC14* encodes an essential Phosphatidylinositol/phosphatidylcholine transfer protein (PITP) and was originally identified in a classical screen for mutations of genes required for the proper function of the yeast secretory pathway (Novick *et al.*, 1980; Bankaitis *et al.*, 1990). The mechanism by which PtdIns4P levels are regulated by Sec14p is unknown (Fairn *et al.*, 2007). Subsequent studies showed that *sac1-22*, which suppressed the growth defects of *sec14-3*, caused an increase in PtdIns4P (Stock *et al.*, 1999). Sac1p is a phosphoinositide 4-phosphatase that can act on several PtdInsPs including PtdIns4P (Guo *et al.*, 1999). Overexpression of *PIK1*, but not *STT4* or *VPS34* could rescue the growth defects of the *sec14-3* mutant at the non-permissive temperature (Hama *et al.*, 1999). These results indicated that PtdIns4P played an important role in secretion in yeast. *sac1Δ* mutants display excessive transport of Chs3p chitin synthase to the cell wall causing cell wall defects, an effect that is also observed in cells overexpressing *PIK1*, but not in cells overexpressing *STT4* (Schorr *et al.*, 2001). Also, at the restrictive temperature, *pik1* temperature-sensitive alleles (*pik1-12*, *pik1-63*, *pik1-83* and *pik1-101*) display a decrease in PtdIns4P production which in turn affects the secretion of invertase and Hsp150p (Garcia-Bustos *et al.*, 1994; Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). These results suggest that Pik1p generated PtdIns4P is necessary for some step in Golgi-to-plasma membrane transport.

pik1-101 causes defects in the actin cytoskeleton and secretion at the Golgi (Walch-Solimena and Novick, 1999). Sequence analysis of the *pik1-101* allele revealed a serine to phenylalanine missense mutation at residue 1045. Electron micrographs

revealed the accumulation of Golgi structures known as Berkeley bodies and vacuole fragmentation which also resulted in the delayed transport of the vacuole enzyme CPY indicating blockage in Golgi-to-cell-surface trafficking and endosomal transport to the vacuole, respectively (Walch-Solimena and Novick, 1999). Berkeley bodies were also observed in cells with a *frq1* temperature-sensitive allele (*frq1-1*) consistent with the idea that Frq1p acts to directly modulate Pik1p PtdIns 4-kinase activity (Strahl *et al.*, 2005). Pik1p was also isolated in a screen to identify mutants which show synthetic lethality in the presence of Gdi1p ectopic expression. Gdi1p is a GDP-dissociation inhibitor for the Sec4p GTPase which regulates secretion to the plasma membrane (Walch-Solimena and Novick, 1999). Further analysis showed that *pik1-101* showed synthetic sickness or lethality with other mutants defective for the late stages of ER-Golgi and intra-Golgi traffic, but not early Golgi transport genes (Walch-Solimena and Novick, 1999). These late acting mutants are *sec7-1*, *bet3-2* and *ypt1-1*. Additionally, *pik1-101* genetically interacted with alleles associated with the actin cytoskeleton such as actin (*act1-2*), type V myosin (*myo2-66*), and profilin (*pfy1-III*).

Several proteins require functional Pik1p and thus, PtdIns4P to become recruited to the Golgi *in vivo*. One such protein is Kes1p, a conserved member of the OSBP family, also termed Osh in yeast (Fang *et al.*, 1996; Li *et al.*, 2002; Jiang *et al.*, 1994). OSBPs have been implicated in the maintenance of sphingolipid and sterol composition in membranes, although their function is poorly understood (Jiang *et al.*, 1994; Beh *et al.*, 2001; Ridgway *et al.*, 1998). A yeast strain deleted for *KES1*, which is non-essential for yeast cell viability, genetically interacted with *SEC14* and was therefore implicated in the formation of Golgi derived vesicles (Bankaitis *et al.*, 1990;

Fang *et al.*, 1996). Kes1p binds PtdIns4P and PtdIns(4,5)P₂ *in vitro*, and this interaction depends on the presence of its PH domain (Li *et al.*, 2002). Full-length Kes1p-GFP dissociates from the Golgi in *pik1-101* cells, but not in *stt4 (stt4-4)* temperature-sensitive cells, or *mss4-2* cells upon shift to the restrictive temperature. The *kes1Δ* mutant partially suppressed the *pik1-101* phenotype at the semi-permissive temperature, but not in an *stt4* temperature-sensitive strain. This suggests that Kes1p may be a negative regulator of Pik1p. These results could be explained if Pik1p derived PtdIns4P is needed for the activation of Gcs1p or Age2p, two GTPase activating proteins (GAPs) for Arf GTPases (Li *et al.*, 2002; Yanagisawa *et al.*, 2002). In line with this hypothesis, *ARF1* genetically interacts with *PIK1* (Levine and Munro, 2002; Godi *et al.*, 2004).

The loss-of-function of *SEC14* can be bypassed by mutations in *CKI1*, an enzyme involved in the CDP-choline pathway for phosphatidylcholine synthesis, or *KES1*, an oxysterol binding protein family member that binds PtdIns4P and sterols (Fairn *et al.*, 2007). A high throughput method for analyzing genetic interactions with a large number of viable deletion mutants, termed synthetic genetic analysis, was performed to determine if the CDP-choline pathway in which *SEC14* is involved, is distinct from the *KES1* pathway (Fairn *et al.*, 2007). Different sets of gene deletions genetically interacted with the *sec14^{ts} cki1* and *sec14^{ts} kes1* double-mutant strains suggesting that both these pathways were distinct. The *sec14^{ts} cki1* double-mutant strain showed genetic interactions with genes encoding the Rab GTPase Ypt31p, as well as members of the TRAPII complex, Trs33p, Trs65p and Trs85p (Sacher *et al.*, 2001; Sciorra *et al.*, 2005). These three subunits comprise the non-essential subunits of the TRAPII complex which acts as a GTP exchange factor for Ypt31p and Ypt32p. Ypt32p

is highly similar in sequence to Ypt31p, but is not functionally redundant with Ypt31p since Ypt32p did not show genetic interactions with *sec14^{ts} cki1* cells. Kes1p is present in *sec14^{ts} cki1* cells suggesting that the Ypt31p/TRAPII proteins function downstream of Kes1p and that Kes1p inhibits the Ypt31p/TRAPII complex (Fairn *et al.*, 2007). Indeed, inactivation of *KES1* in *sec14^{ts} cki1 ypt31* or *sec14^{ts} cki1 trapII* cells restored growth. Also, since *KES1* is present in *sec14^{ts} cki1* cells and has been demonstrated to bind PtdIns4P, Kes1p and Pik1p may control vesicular transport through the regulation of Golgi PtdIns4P. Inactivation of *KES1* in *pik1^{ts}* cells restored growth and secretion defects associated with the inactivation of *PIK1*. Inactivation of *KES1* did not restore the growth of *stt4^{ts}* cells suggesting that *KES1* does not regulate the function of the *STT4* generated pool of PtdIns4P. Cells deleted for both *kes1* and *frq1* were also viable in keeping with the role of Frq1p in activating Pik1p. Kes1p regulates PtdIns4P levels since *kes1*Δ cells have increased levels of PtdIns4P. PtdIns4P levels were restored to wild-type in *kes1 pik1^{ts}* cells at the non-permissive temperature, since *pik1^{ts}* cells have reduced PtdIns4P at the restrictive temperature. This was corroborated by a PH_{Osh2p}-GFP probe which monitored PtdIns4P at the Golgi. In *KES1* inactivated cells, there was an increase in the association of PH_{Osh2p}-GFP at the Golgi. Also, the probe was reestablished at the Golgi in *pik1^{ts} kes1* cells (Fairn *et al.*, 2007).

PtdIns4P pools are also regulated by the synaptojanin-like phosphoinositide phosphatases Inp51/Sjl1, Inp52/Sjl2 and Inp53/Sjl3 (Nguyen *et al.*, 2005). These proteins are homologous to mammalian PtdIns(4,5)P₂ 5-phosphatases. *PIK1* displays a synthetic lethal interaction with the *INP53/SJL3* deletion mutant and a *pik1* temperature-sensitive allele exacerbates the phenotype of *INP51/SJL1* or *INP52/SJL2*

deletion mutants. *inp51Δ/sjl1Δ pik1^{ts}* and *inp52Δ/sjl2Δ pik1^{ts}* display reduced PtdIns4P (29% and 11%, respectively), but increased PtdIns(4,5)P₂ levels (62% and 19%, respectively). The double-mutants also display more severe defects in invertase secretion than the *pik1^{ts}* cells alone. These results are consistent with previous evidence suggesting that PtdIns4P rather than PtdIns(4,5)P₂ is required for Golgi-to-plasma membrane trafficking of secretory proteins in *S. cerevisiae*.

The *pik1-139* conditional strain was shown to genetically interact with the non-essential deletion strains of *DRS2*, *TRS33*, *KRE11* and *YPT31* (Sciorra *et al.*, 2005). These genetic interactions were specific to *PIK1* because a genetic interaction was not observed with *STT4* or *MSS4*. Ypt31p encodes a Rab11-related small GTPase that is essential for secretion in yeast (Benli *et al.*, 1996; Jedd *et al.*, 1997). Rab11 is recruited to the Golgi in a manner that depends on PtdIns4KIIIβ (de Graaf *et al.*, 2004). The Pik1p orthologue in *Arabidopsis*, AtPI4Kβ1 associates with the Rab11 orthologue AtRabA4, mediated by the AtPI4Kβ1 NHD (Preuss *et al.*, 2006). Interestingly, Ypt32p, another Rab GTPase that is 81% identical to Ypt31p and thought to be genetically redundant with Ypt31p, did not show synthetic phenotypes with *pik1-139* (Sciorra *et al.*, 2005). Drs2p is an aminophospholipid translocase (Chen *et al.*, 1999). Kre11p and Trs33p encode non-essential subunits of a large Golgi-associated complex made up of 10 subunits called the TRAPP II complex (Sacher *et al.*, 2001). Cells carrying mutations in the essential components of this complex display defects in secretion as well as aberrant Golgi derived structures (Sacher *et al.*, 2001). The double-mutant *ypt31Δ pik1-139* cells displayed Berkeley bodies in the cytoplasm. Also, plasma membrane proteins that normally continuously recycle back to the Golgi apparatus via early endosomes

(Chs3p and Snc1p) were mislocalized (Sciorra *et al.*, 2005). *ypt31-101 ypt32Δ* cells did not show any changes in PtdIns4P levels at the restrictive temperature and Pik1p was still localized to the Golgi indicating that Ypt31p was not necessary to activate Pik1p or assist in the Golgi recruitment of Pik1p *in vivo* (Sciorra *et al.*, 2005).

Gavin *et al.*, (2002) performed high throughput proteomic studies using tandem affinity purification (TAP) and mass spectrometry to identify multi-protein complexes of many proteins in *S. cerevisiae*. Amongst the numerous proteins which were genomically TAP-tagged was Bmh2p, a multifunctional protein homologous to 14-3-3 proteins in mammalian cells (van Heusden and Steensma, 2006, for review). 14-3-3 proteins are acidic dimers (*BMH1* and *BMH2* in *S. cerevisiae*) and are involved in a variety of cellular processes such as signaling, apoptosis, exocytosis, transcriptional regulation, cytoskeletal rearrangements, enzyme regulation and cell cycle control (14-3-3 physically interacts with *cdc25*) (Conklin *et al.*, 1995). Amongst the proteins pulled-down with *BMH2* was *PIK1* and others including *BMH1*, *NTH1*, *NTH2*, *RTG2* and *PSK1*. Both the *NTH1* and *NTH2* genes encode neutral trehalase proteins involved in breaking down cytoplasmic trehalose in *S. cerevisiae*. *RTG2* is a gene whose protein product induces the formation of extrachromosomal ribosomal DNA circles (ERCs) involved in yeast cell longevity. *PSK1* is a gene encoding a protein involved in glycogen synthesis (Nwaka and Holzer, 1998; Rutter *et al.*, 2002). It is still unclear what the role of *PIK1* is in this complex, but suggests that Pik1p is involved in many cellular processes, some of which are still unknown.

Most recently, a paper was published documenting the physiologically relevant interaction between Pik1p and 14-3-3 proteins in *S. cerevisiae* (Demmel *et al.*, 2008).

Bmh1p and Bmh2p are redundant proteins when deleted alone, but are essential in combination (Dougherty and Morrison, 2004). Using yeast two-hybrid protein-protein interaction studies, Demmel *et al.* (2008) confirmed the Pik1p interaction with Bmh1p and also Bmh2p, both of which are highly similar in amino acid sequence.

Furthermore, these interactions were approximately 10-fold weaker than the interaction between Pik1p and Frq1p (Demmel *et al.*, 2008). The interaction with Pik1p and Bmh2p was lost when the C-terminal catalytic domain of Pik1p was deleted. GST-pull-down experiments also confirmed the interaction between Pik1p and the 14-3-3 proteins (Demmel *et al.*, 2008). It was then of interest to see if Pik1p formed a common complex with Frq1p and the 14-3-3 proteins. Indeed, glycerol velocity gradient centrifugation showed that all 4 proteins, Pik1p, Frq1p, Bmh1p and Bmh2p, formed a heterotetrameric complex. Could the interaction between Pik1p and the 14-3-3 proteins play a role in secretion? No synthetic genetic interaction was observed between *bmh1Δ* or *bmh2Δ* with *pik1-101*. However, there was an enhancement of the growth defect of *pik1-101* cells by overexpressing each of *BMH1* and *BMH2* (Demmel *et al.*, 2008).

Indeed, the overexpression of *BMH1* and *BMH2* enhanced the growth defects of mutants of genes involved in TGN to plasma membrane transport (*sec1-1*, *sec2-41*, *sec3-2*, *sec4-8*, *sec15-1*) and mutants of genes involved in Golgi vesicle formation (*arf1Δ*, *vps1Δ*, *ypt31Δ* *ypt32ts* and *sec14-3*). Overexpression of *BMH1* and *BMH2* also exacerbated the growth defects of mutants of genes involved in the cytoskeleton and polarized transport of secretory vesicles (*act1-2* and *myo2-66*). These data suggest that the function of 14-3-3 proteins may be to counteract Pik1p function (Demmel *et al.*, 2008). The use of a mutated GFP-tagged general amino acid permease (Gap1p^{mut})

which lacks sites for ubiquitination is used to visually assay for exocytosis. The mutation ensures that Gap1p is not degraded once it gets to the plasma membrane. In cells overexpressing *BMH2*, exocytosis of Gap1p^{mut} was reduced. This phenotype phenocopied the *pik1-101* mutant in Gap1p^{mut} exocytosis. Fractionation experiments showed that the interaction between Pik1p and the 14-3-3 proteins occurred in the cytoplasm and not in the membrane fraction. In *bmh1Δ bmh2ts* mutants at 25°C, there was an increase in Pik1p in the nucleus and a decrease in Pik1p at the TGN (Demmel *et al.*, 2008). Thus, based on the redistribution of Pik1p, the 14-3-3 interaction may be required for the maintenance of a pool of Pik1p in the cytoplasm making it more accessible to the TGN.

14-3-3 proteins often bind to their targets through phosphorylated residues (Dougherty and Morrison, 2004). It was then of interest to see if the Pik1p-14-3-3 interaction was regulated in a similar manner. Pik1p-TAP bound to either GST-Bmh1p or GST-Bmh2p only in the presence of phosphatase inhibitors and not in the presence of lambda protein phosphatase indicating that the interaction was dependent on the phosphorylation of Pik1p (Demmel *et al.*, 2008). Tandem mass spectrometry showed that 4 regions of Pik1p were phosphorylated and two of these regions matched 14-3-3 binding motifs (S396 and S605). Thus, mutants of these phosphorylated residues were made and tested for their interaction with Bmh1p and Bmh2p using yeast two-hybrid assays. Through these studies, it was shown that S396 is necessary, but not sufficient for the interaction since deletion of the Pik1p catalytic domain abrogated the interaction between Pik1p and the 14-3-3 proteins (Demmel *et al.*, 2008). This suggests that

additional structural requirements in the C-terminal region of Pik1p are required for its interaction with 14-3-3 proteins.

Pik1p was shown to rapidly and reversibly relocate from the nucleus to the cytoplasm upon a secretion block (i.e., in a *sec6-4* mutant) (Demmel *et al.*, 2008). This arrest of secretion response (ASR) occurs in most *sec* mutants and also results in the relocation of other nuclear proteins most likely due to nuclear import inhibition. Pik1p phosphorylation was increased during ASR suggesting that phosphorylation plays a role in Pik1p distribution (Demmel *et al.*, 2008). Under conditions of nutrient limitation, Pik1p also relocated from the TGN to the nucleus. In cells grown in the absence of glucose, there was an increase in GFP-Pik1p in the nucleus and cytoplasm and reduced labeling at the TGN (Demmel *et al.*, 2008). Furthermore, after glucose deprivation, phosphorylated Pik1p was prevalent and an increase in Pik1p-Bmh1p complex formation was observed. The relocalization of Pik1p to the nucleus and cytoplasm might be a way for cells to quickly adjust PtdIns4P production and vesicle formation at the TGN to growth conditions. In cells overexpressing *BMH1* and *BMH2*, Pik1p was found in the cytoplasm and was no longer found in the nucleus, whereas the TGN localization was unchanged (Demmel *et al.*, 2008). Thus, 14-3-3 levels appear to determine the amount of Pik1p kept in the cytoplasm. Finally, the phosphorylation or interaction with 14-3-3 proteins did not have an effect on Pik1p lipid kinase activity (Demmel *et al.*, 2008). Thus, nucleo-cytoplasmic shuttling of Pik1p is regulated by 14-3-3 proteins and coordinates Golgi function with cell growth.

Pik1p may also be involved in endocytosis. Membrane protein uptake was followed by monitoring the rate of degradation of an integral membrane protein and

ABC transporter, Ste6p, by pulse-chase analysis (Audhya *et al.*, 2000). Endocytosis was also monitored using the lipophilic dye FM4-64. At the restrictive temperature, *pik1^{ts}* (*pik1-83* and *pik1-101*) cells were unable to degrade Ste6p and also failed to accumulate FM4-64 in the vacuolar membrane (Audhya *et al.*, 2000; Walch-Solimena and Novick, 1999). Thus, Pik1p is required for plasma membrane-to-vacuole transport.

PtdIns4P has also been implicated in the process of pexophagy, the selective degradation of peroxisomes (Dunn *et al.*, 2005; Sakai *et al.*, 2006). The integration of the peroxisome into the vacuole requires the transient formation of a cup-shaped double membrane structure called the MIPA (*micropexophagy specific membrane apparatus*) during micropexophagy. *Pichia pastoris* is a methylotrophic yeast whereby the formation of peroxisomes are induced when the yeast is grown in the presence of methanol (Sakai *et al.*, 2006). Pexophagy is then induced when the cells are shifted to growth in media with glucose. During the process of engulfment of peroxisomal structures by the vacuole, a protein complex forms at a site juxtaposed to the vacuole termed the *pre-autophagosomal structure* (PAS). PpAtg26p has a GRAM (glucosyltransferase, Rab-like GTPase activators and myotubularins) domain which binds PtdIns4P. PpAtg26p is targeted to the PtdIns4P enriched MIPA through its GRAM domain at the PAS (Yamashita *et al.*, 2006). PpPik1p is mainly responsible for the production of PtdIns4P at the MIPA, and PpLsb6p also contributes to this pool, but to a lesser extent than PpPik1p. Furthermore, PpAtg26p was not recruited to the PAS in cells with a genomically integrated lipid kinase dead PpPik1p allele (PpPik1p S994F). PpPik1p or PpLsb6p did not colocalize with the MIPA suggesting that PtdIns4P may be transported from its production site to the MIPA. A kinase dead version of PpStt4p

(PpStt4p S1818F) did not contribute to micropexophagy in *P. pastoris* (Yamashita *et al.*, 2006).

In summary, Pik1p is localized to 3 compartments in the cell: (A) the *trans*-Golgi; (B) the nucleus, and (C) the cytoplasm. Pik1p through the PtdIns4P it produces as well as through protein-protein interactions, regulates membrane trafficking processes, such as secretion, vacuolar trafficking and endocytosis.

1.2.3.2.3. Stt4p

Finally, the third PtdIns 4-kinase in *S. cerevisiae* is Stt4p for staurosporine and temperature-sensitive (Yoshida *et al.*, 1994). Staurosporine is an antifungal alkaloid isolated from *Streptomyces sp.* that was initially thought to inhibit some protein kinase C (PKC)-related enzymes (Omura *et al.*, 1977). Pkc1p is the only PKC-related protein kinase in yeast which plays an important role in the cell wall integrity pathway (Levin *et al.*, 1990; Paravicini *et al.*, 1992). Pkc1p is an upstream activator of a MAP kinase cascade comprised of the MEKK Bck1p, the Mkk1p and Mkk2p MEKs and a MAP kinase Slt2p/Mpk1p (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993; Martin *et al.*, 1993). Pkc1p is activated by its interaction with the essential small GTPase Rho1p and likely Rho2p (Madaule *et al.*, 1987; Nonaka *et al.*, 1995). Rho1p and Rho2p are activated by the guanine nucleotide exchange factors (GEFs) Rom1p and Rom2p, respectively (Ozaki *et al.*, 1996). The Pkc1p-mediated activation of the Slt2p MAP kinase cascade was shown to be initiated by two transmembrane receptor-like proteins Wsc1p and Mid2p, which both interact with Rom2p (Ozaki *et al.*, 1996; Heinisch *et al.*, 1999; Philip and Levin, 2001). The staurosporine sensitive phenotype of *stt4^{ts}* (*stt4-1*)

cells can be suppressed by overexpression of Pkc1p, suggesting that Stt4p functions upstream of Pkc1p (Yoshida *et al.*, 1994). Overexpression of Pkc1p rescues the staurosporine sensitivity, but not temperature sensitivity of *stt4^{ts}* mutants (one being *stt4-1*) suggesting additional cellular functions for Stt4p (Yoshida *et al.*, 1994).

Mss4p (multicopy suppressor of *STT4*) is the only PtdIns4P 5-kinase in *S. cerevisiae* and was cloned as a multicopy suppressor of the *stt4-1* mutation suggesting that Mss4p functions downstream of Stt4p in the same pathway (Yoshida *et al.*, 1994). Stt4p localizes to the plasma membrane where it generates a pool of PtdIns4P that is converted to PtdIns(4,5)P₂ by Mss4p. This leads to the activation of the Pkc1p-mediated MAP kinase cascade. Stt4p and Mss4p-dependent PtdIns(4,5)P₂ generation is required for Rom2p to become recruited to the plasma membrane (Audhya and Emr, 2002). It was shown that the bulk of PtdIns(4,5)P₂ generated at the plasma membrane requires functional Stt4p, but not Pik1p (Levine and Munro, 2002; Roy and Levine, 2004; Perera *et al.*, 2004).

In addition to the PH domain of Stt4p which aids in the recruitment of Stt4p to the plasma membrane, Stt4p interacts with Sfk1p, a multicopy suppressor of the *stt4-4* allele, but not the *pik1-83* allele (Audhya and Emr, 2002). Sfk1p is an integral membrane protein that colocalizes with Stt4p to small punctate spots at the plasma membrane. In cells lacking Sfk1p, Stt4p localization and PtdIns4P production at the plasma membrane is reduced. Thus, the PH domain of Stt4p alone is not sufficient for its maintenance at the plasma membrane (Levine and Munro, 1998).

Stt4p also has a role in maintaining the actin cytoskeleton. At the restrictive temperature, *stt4-1* or *mss4-2* cells have defects in the actin cytoskeleton, unlike the

pik1-83 mutation (Audhya *et al.*, 2000). The actin cytoskeletal defects can be suppressed by inactivation of *SAC1* (by *sac1Δ* or *sac1-23*) suggesting a genetic interaction between the two proteins (Foti *et al.*, 2001). *SAC1* null cells and *sac1-23* cells display an 8 to 12-fold increase in intracellular PtdIns4P which can be restored to wild-type levels by an *stt4* temperature-sensitive mutant (*stt4-4*), but not by the *pik1* (*pik1-83*) or *lsb6* (*lsb6Δ*) mutants (Foti *et al.*, 2001; Tahirovic *et al.*, 2005).

Stt4p is also required for the recruitment of Cla4p, a p21-activated kinase (PAK) to sites of polarized cell growth (Wild *et al.*, 2004). This is independent of its function with Mss4p, but requires the Rho-type small GTPase Cdc42p. Cdc42p is a multifunctional protein involved in many different processes such as the regulation of the mating pheromone and stress response MAP kinase cascade, septin assembly at the bud neck, and polarization of the actin cytoskeleton (Adams *et al.*, 1990; Richman *et al.*, 2002; Raitt *et al.*, 2000; Gladfelter *et al.*, 2002; Lamson *et al.*, 2002). It also drives exit from mitosis and is involved in vacuolar docking and fusion (Eitzen *et al.*, 2001; Muller *et al.*, 2001; Eitzen *et al.*, 2002; Hofken and Schiebel, 2002; Seshan *et al.*, 2002; Chirolì *et al.*, 2003). The Cla4p PAK is a direct interactor of Cdc42p and is required for polarized cell growth. Cla4p binds to Cdc42p through its p21-binding domain and to phosphoinositides by its PH domain (Cvrckova *et al.*, 1995; Wild *et al.*, 2004). PtdIns4P is required for Cla4p recruitment to areas of polarized growth generated by Stt4p (Wild *et al.*, 2004). Cla4p is mislocalized in cells carrying the temperature-sensitive *stt4-4* allele at the restrictive temperature, but not in cells with a temperature-sensitive *pik1-83* or *mss4-102* allele. Thus, Cla4p serves as a coincidence sensor for the Stt4p generated pool of PtdIns4P and Cdc42p at the plasma membrane.

In summary, Stt4p localizes to the plasma membrane in *S. cerevisiae*, and produces the PtdIns4P precursor for PtdIns(4,5)P₂ at the plasma membrane. At the plasma membrane, PtdIns(4,5)P₂ is required for polarized growth and organization of the actin cytoskeleton.

1.2.3.3. PtdIns 4-kinases in *S. pombe*

S. pombe Pik1p is an 851 amino acid protein with predicted molecular weight of 97 kDa (Wood *et al.*, 2002). It is located on chromosome I, locus SPAC22E12.16c, and the genomic sequence has 3 introns. It has since been named *pik1*, after its orthologue in *S. cerevisiae*. Pik1p is one of three PtdIns 4-kinases in *S. pombe* similar to Pik1p in budding yeast. The two other PtdIns 4-kinases that have been annotated in *S. pombe* are a larger PtdIns 4-kinase of 214 kDa (orthologous to Stt4p in *S. cerevisiae*, the SPB577.06c locus in *S. pombe*) and a smaller one of 72 kDa (orthologous to Lsb6p in *S. cerevisiae*, the SPAC343.19c locus in *S. pombe*). The *pik1* gene is not transcriptionally regulated during the haploid cell cycle as indicated in the Rustici *et al.* (2004) paper reporting genomic transcription levels of many genes throughout the cell cycle. However, a sharp increase in *pik1* transcript levels during meiosis is observed suggesting a major role for *pik1* in sporulation/germination in *S. pombe* (Mata and Bahler, 2003).

The cloning and characterization of the *S. pombe* orthologue of *FRQ1*, *ncs1*, was published in 2004 (Hamasaki-Katagiri *et al.*, 2004). The localization of chromosomally integrated Ncs1p-GFP in haploid cells is cytoplasmic and punctate with string-like patterns predominantly along the plasma membrane. Co-localization studies

with Pik1p have not been performed, but it is likely that the punctate staining may be Golgi and associated with Pik1p. Hamasaki-Katagiri (2004) report that *ncs1* regulates sporulation and binds calcium. For example, the *ncs1* partial deletion of the C-terminus shows spontaneous sporulation in a nutrient-independent manner and the *ncs1* partial deletion is lethal in the presence of high calcium concentrations (0.1 M CaCl₂). Ncs1p has four EF-hand motifs of which three are able to bind Ca²⁺ based on the primary structure (Hamasaki-Katagiri *et al.*, 2004). Furthermore, analysis of Ncs1p mutants that cannot bind calcium and cannot be myristoylated showed that the calcium-binding activity of *ncs1* was essential for both the sporulation and calcium-sensitivity phenotypes while myristoylation only mildly effected these phenotypes (Hamasaki-Katagiri *et al.*, 2004). Interestingly, *ncs1* is greatly upregulated in high concentrations of calcium. Hamasaki-Katagiri also report that Ncs1p binds tightly to the N-terminal region of *S. cerevisiae* Pik1p. It has not yet been shown to bind to *S. pombe* Pik1p. Thus, it is likely that *ncs1* interacts with *S. pombe* Pik1p, binds calcium and is myristoylated. It is possible that its association with Pik1p confers calcium regulated activity and/or association with some membrane compartment.

Pik1p in *S. pombe* is essential for haploid cell viability. It localizes to the Golgi and to the medial region late in cytokinesis after contractile ring formation, but at about the same time as septation (Park, 2007). A *pik1* loss-of-function mutant, at the restrictive temperature, results in cells that cannot separate with abnormally thick and supernumerary septa, while contractile ring formation remained normal (Park, 2007). The implications of these results are discussed in greater detail in chapter 5, the discussion.

Chapter 2: Rationale, hypotheses and experimental approaches

Earlier studies in this laboratory suggested that Cdc4p, a contractile ring protein essential for cytokinesis, interacted with a PtdIns 4-kinase, Pik1p (Desautels *et al.*, 2001). This interaction was unexpected and unusual in that one of the established functions of Cdc4p is that of a myosin essential light chain. However, more recent work supports the hypothesis that Cdc4p may have more than one function. Thus, the main objective of this research work was to establish if Pik1p plays a role in cytokinesis in *S. pombe* and to determine the importance of its interaction with Cdc4p in cytokinesis.

2.1. Hypotheses

- 1) Pik1p lipid kinase activity is essential for cytokinesis in *S. pombe*;
- 2) Cdc4p interacts with Pik1p to affect its localization and/or activity required for cytokinesis.

2.2. Specific objectives

- i) To acquire and clone the *pik1* cDNA into bacterial and yeast expression vectors;
- ii) To identify an amino acid residue essential for lipid kinase activity in Pik1p;
- iii) To selectively alter the Pik1p protein to abolish lipid kinase activity;
- iv) To evaluate the effects of loss of Pik1p lipid kinase activity on cytokinesis;
- v) To identify an amino acid residue in Pik1p required for its interaction with Cdc4p;
- vi) To selectively alter the Pik1p protein to abolish the Pik1p interaction with Cdc4p;
- vii) To evaluate the effects of expression of mutated Pik1p that lacks Cdc4p binding activity on cytokinesis.

2.3. Experimental approaches

Sequence alignments of several lipid kinases were used to identify conserved motifs in the *pik1* sequence that might be required for lipid kinase activity. Cdc4p is known to bind the IQ-motifs in type II myosins. Therefore, the Pik1p protein was searched for putative IQ-motifs. Site-directed mutagenesis was used to replace each of the individual conserved amino acid residues presumed to be important for Pik1p lipid kinase activity and Cdc4p-binding activity to alanine. Verification of the effectiveness of these mutations was performed by ectopically expressing the wild-type and mutant enzymes, followed by measurements of lipid kinase activity and Cdc4p binding activity

with yeast two-hybrid assays and ELISAs. To evaluate the importance of Pik1p lipid kinase activity and Cdc4p binding activity in cytokinesis in *S. pombe*, two approaches were used: (1) ectopic expression of the *pik1* wild-type and mutated enzymes in *S. pombe* cells; and (2) allele replacement of the wild-type genomic *pik1* sequence with the mutated versions of *pik1*.

Ectopic expression is a useful tool for studying the function of proteins *in vivo*. Ectopic expression of a catalytically inactive protein is expected to displace the wild-type enzyme disrupting the normal function of the protein. Likewise, ectopically expressing an enzyme that may be improperly localized in the cell can provide clues as to the importance of the disrupted cellular pathway. *S. pombe* cells carrying ectopic expression constructs of each of the *pik1* alleles were assessed for their ability to accumulate in the cells, for their lipid kinase activity, their effect on cell proliferation and cell morphology.

Finally, allele replacement of the *pik1* wild-type sequence with mutant forms was used to study the effects of expressing the mutant genes under the control of the *pik1* native promoter. Homologous recombination was used to introduce the mutations known to abolish Pik1p lipid kinase activity and Cdc4p binding activity into the chromosomal *pik1* locus. Tetrad analysis was then used to assess the viability of the cells that carry the *pik1* mutations.

Chapter 3: Materials and methods

3.1. *S. pombe* strains, cultures and genetics

3.1.1. *S. pombe* strains, media and cultures

The genotypes and sources of *S. pombe* strains used in this study are listed in Table 3.1. Standard media used for cultivation of *S. pombe* were YES (yeast extract with supplements), EMM (Edinburgh minimal medium) and ME (malt extract). The pH of EMM was adjusted to 5.5 using glacial acetic acid to optimize cell growth. Supplements for EMM included lysine, leucine, adenine, uracil and histidine, each at 225 mg/L (Moreno *et al.*, 1991). Agar was added to 2% (w/v) to make solid media. For *S. pombe* haploid cells carrying pREP plasmids, cells were cultured in EMM lacking leucine in the presence or absence of 15 μ M thiamine. Thiamine was used to repress gene expression from the *nmt1* promoter of the pREP plasmids (Maundrell, 1990; Maundrell, 1993; Basi *et al.*, 1993). Diploid strains made from haploids carrying the *ade6-M210* and *ade6-M216* alleles were cultured in EMM lacking adenine because of the complementation of the *ade6-M216* and *ade6-M210* alleles.

Table 3.1.List of *S. pombe* strains used in this study

Strain Number	Genotype	Source
N2	<i>h⁺ leu1-32 ura4-D18 ade6-210</i>	P. Nurse
N3	<i>h⁻ leu1-32 ura4-D18 ade6-216</i>	P. Nurse
N1184	<i>h⁻ cdc4^{R33K} leu1-32 ade6-210 ura4-D18</i>	M. Balasubramanian
N1186	<i>h⁻ cdc4^{F12L} leu1-32 ade6-210 his3-D1</i>	M. Balasubramanian
N1187	<i>h⁻ cdc4^{G82D} leu1-32 his3-D1</i>	M. Balasubramanian
N1185	<i>h⁺ cdc4^{G107S} leu1-32 ade6-210 ura4-D18</i>	P. Nurse
N1188	<i>h⁺ cdc4^{G19E} leu1-32 ade6-210 ura4-D18</i>	P. Nurse
N1477	N2 pREP1	This study
N1174	N2 pREP1- <i>pik1</i>	This study
N1172	N2 pREP41- <i>pik1</i>	This study
N1176	N2 pREP81- <i>pik1</i>	This study
N1375	N2 pREP1- <i>pik1</i> ^{D709A}	This study
N1376	N2 pREP41- <i>pik1</i> ^{D709A}	This study
N1377	N2 pREP81- <i>pik1</i> ^{D709A}	This study
N1273	N2 pREP1- <i>pik1</i> ^{R838A}	This study
N1175	N2 pREP41- <i>pik1</i> ^{R838A}	This study
N1177	N2 pREP81- <i>pik1</i> ^{R838A}	This study
N1296	N2 pREP1- <i>pik1</i> ^{D709A, R838A}	This study
N1297	N2 pREP41- <i>pik1</i> ^{D709A, R838A}	This study
N1230	N2 pREP81- <i>pik1</i> ^{D709A, R838A}	This study

N1276	N1185 pREP1	This study
N1281	N1185 pREP1- <i>pikI</i>	This study
N1277	N1185 pREP41- <i>pikI</i>	This study
N1278	N1185 pREP1- <i>pikI</i> ^{D709A}	This study
N1282	N1185 pREP41- <i>pikI</i> ^{D709A}	This study
N1279	N1185 pREP1- <i>pikI</i> ^{R838A}	This study
N1283	N1185 pREP41- <i>pikI</i> ^{R838A}	This study
N1280	N1185 pREP1- <i>pikI</i> ^{D709A, R838A}	This study
N1284	N1185 pREP41- <i>pikI</i> ^{D709A, R838A}	This study
N1262	N1188 pREP1	This study
N1263	N1188 pREP1- <i>pikI</i>	This study
N1267	N1188 pREP41- <i>pikI</i>	This study
N1264	N1188 pREP1- <i>pikI</i> ^{D709A}	This study
N1268	N1188 pREP41- <i>pikI</i> ^{D709A}	This study
N1265	N1188 pREP1- <i>pikI</i> ^{R838A}	This study
N1269	N1188 pREP41- <i>pikI</i> ^{R838A}	This study
N1266	N1188 pREP1- <i>pikI</i> ^{D709A, R838A}	This study
N1270	N1188 pREP41- <i>pikI</i> ^{D709A, R838A}	This study
N1246	N1187 pREP1	This study
N1247	N1187 pREP1- <i>pikI</i>	This study
N1251	N1187 pREP41- <i>pikI</i>	This study
N1248	N1187 pREP1- <i>pikI</i> ^{D709A}	This study
N1252	N1187 pREP41- <i>pikI</i> ^{D709A}	This study

N1249	N1187 pREP1- <i>pikI</i> ^{R838A}	This study
N1253	N1187 pREP41- <i>pikI</i> ^{R838A}	This study
N1252	N1187 pREP1- <i>pikI</i> ^{D709A, R838A}	This study
N1254	N1187 pREP41- <i>pikI</i> ^{D709A, R838A}	This study
N1220	N1186 pREP1	This study
N1224	N1186 pREP1- <i>pikI</i>	This study
N1221	N1186 pREP41- <i>pikI</i>	This study
N1222	N1186 pREP1- <i>pikI</i> ^{D709A}	This study
N1225	N1186 pREP41- <i>pikI</i> ^{D709A}	This study
N1223	N1186 pREP1- <i>pikI</i> ^{R838A}	This study
N1226	N1186 pREP41- <i>pikI</i> ^{R838A}	This study
N1298	N1186 pREP1- <i>pikI</i> ^{D709A, R838A}	This study
N1378	N1186 pREP41- <i>pikI</i> ^{D709A, R838A}	This study
N1286	N1184 pREP1	This study
N1287	N1184 pREP1- <i>pikI</i>	This study
N1291	N1184 pREP41- <i>pikI</i>	This study
N1288	N1184 pREP1- <i>pikI</i> ^{D709A}	This study
N1292	N1184 pREP41- <i>pikI</i> ^{D709A}	This study
N1289	N1184 pREP1- <i>pikI</i> ^{R838A}	This study
N1293	N1184 pREP41- <i>pikI</i> ^{R838A}	This study
N1290	N1184 pREP1- <i>pikI</i> ^{D709A, R838A}	This study
N1294	N1184 pREP41- <i>pikI</i> ^{D709A, R838A}	This study
N1550	<i>h</i> ⁺ / <i>h</i> ⁻ <i>pikI</i> ^{wt} / <i>pikI</i> ^{wt} : <i>term</i> ^{nmt1} : <i>ura4</i> ⁺ <i>ade6-M210/ade6-M216 his3-D1/his3-D1 leu1-</i>	This study

	<i>32/leu1-32 ura4-D18/ura4-D18</i>	
N1565	<i>h⁺/h⁻ pik1^{wt}/pik1^{D709A}:term^{nmt1}:ura4⁺ ade6-M210/ade6-M216 his3-D1/his3-D1 leu1-32/leu1-32 ura4-D18/ura4-D18</i>	This study
N1582	<i>h⁺/h⁻ pik1^{wt}/pik1^{R838A}:term^{nmt1}:ura4⁺ ade6-M210/ade6-M216 his3-D1/his3-D1 leu1-32/leu1-32 ura4-D18/ura4-D18</i>	This study
N1596	<i>h⁺/h⁻ pik1^{wt}/pik1^{D709A, R838A}:term^{nmt1}:ura4⁺ ade6-M210/ade6-M216 his3-D1/his3-D1 leu1-32/leu1-32 ura4-D18/ura4-D18</i>	This study

Plates with *S. pombe* colonies were sealed with parafilm and stored at 4°C for up to 1 month. For indefinite storage of *S. pombe* strains, 850 µL of a stationary phase culture of cells was added to 150 µL of glycerol to a final concentration of 15% in a cryovial, mixed by inversion and stored at -70°C.

3.1.2. Determination of genotype

To determine the genotype of a strain, cells were tested for their ability to grow on plates lacking particular nutritional supplements (Moreno *et al.*, 1991). The auxotrophic markers most commonly used in this study were adenine, histidine, leucine, lysine and uracil. To test for auxotrophy, YES plates with isolated colonies were replica plated onto EMM plates with and without the supplement. For example, to determine if a strain carried the *ade6-M210* or the *ade6-M216* allele, cells were replica plated onto plates containing normal amounts or low amounts of adenine (Moreno *et al.*, 1991). In the presence of low levels of adenine, colonies of cells carrying the *ade6-M210* allele are dark pink and colonies of cells carrying the *ade6-M216* allele are light pink. To test whether a strain was h^+ or h^- , it was crossed with cells of each mating type (tester strains) and the cells scored for their ability to form spores. Colony PCR (Section 3.2.9) was used to verify the introduction of mutant sequences into the *pik1* genomic locus.

3.1.3. *S. pombe* transformation

The yeast cells were transformed using an optimized lithium acetate procedure developed for transformation of *S. cerevisiae* cells, called TRAF0 (Gietz and Woods,

2002). A single colony of haploid or diploid cells was used to inoculate a starter culture of 5 mL of YES or EMM lacking adenine, respectively. Cells were grown overnight at 30°C (or 25°C for temperature-sensitive strains). An aliquot of the starter culture was used to inoculate a 50 mL culture at an initial cell density of 1×10^5 cells/mL. The cells were cultured overnight for 16 hours at 30°C (or 25°C for temperature-sensitive strains). The cells were collected by centrifugation (700 x g for 10 minutes) and resuspended in 360 µL of a solution containing 240 µL of 50% w/v PEG 3500, 36 µL of 1.0 M LiAc, 50 µL of 2 mg/mL salmon sperm single-stranded carrier DNA, and 34 µL of plasmid DNA in distilled, sterilized water. Up to 1 µg of DNA was used for transformation. Cells were then incubated at 42°C for 40 minutes. After collecting the cells by centrifugation (13,000 x g for 1 minute), they were plated onto EMM lacking leucine plates for haploid cells transformed with the pREP plasmids, or EMM lacking adenine and uracil plates for diploids transformed with the integration construct used for the allele replacement studies.

3.1.4. Allele replacement

The *pik1* mutant alleles created by site-directed mutagenesis were integrated into the *pik1* chromosomal locus under the control of the native *pik1* promoter. This approach requires the transformation of diploid cells with linear DNA fragments containing homologous regions 5' and 3' of the mutation. Diploid cells were used because of the possibility that one or more of the alleles were lethal in haploid cells. Following the introduction of the DNA into the diploid cells, the effects of these mutations were tested by tetrad analysis (Moreno *et al.*, 1991). Tetrad analysis was

used to determine the viability of individual spores dissected from an ascus, which contains four spores, hence the tetrad.

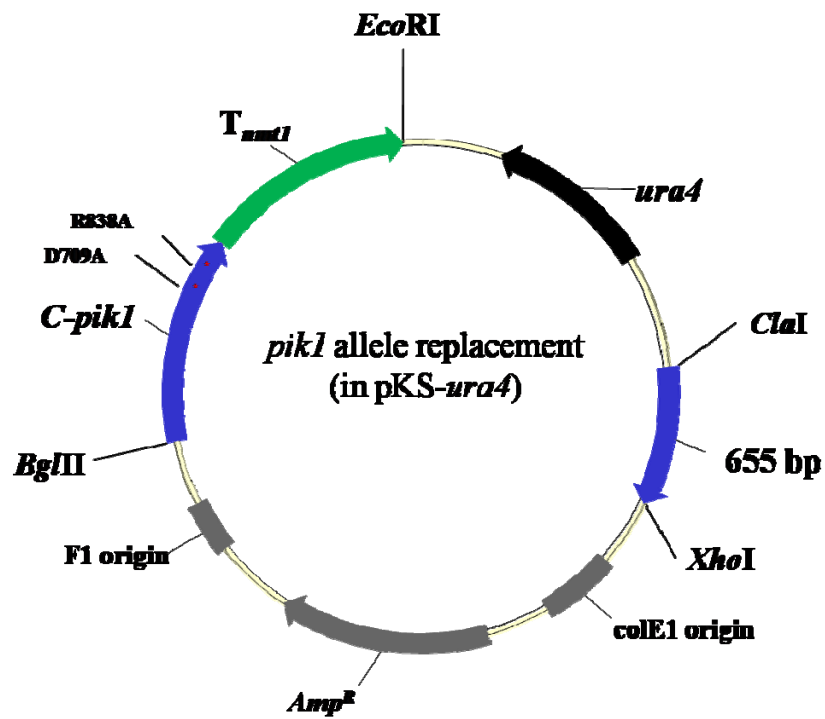
3.1.4.1. Plasmids for allele replacement

The plasmid pKS-*ura4* was used for the construction of the allele replacement constructs (Bahler *et al.*, 1998b). The pKS-*ura4* plasmid was modified to include a *Bgl*II site in the multiple cloning region. This was performed by PCR mutagenesis. In order to replace the wild-type genomic *pik1* gene with a mutated sequence by homologous recombination, it is necessary that the mutated sequence in the integration construct carry regions of homology both upstream and downstream of the mutation(s). For efficient homologous recombination to occur, approximately 300 bp to 1 Kb of sequence should be used (Bahler *et al.*, 1998b). The recombinant DNA fragment that was used to introduce the mutant alleles into the *pik1* chromosomal locus is shown in Figure 3.1. The region C-*pik1* T_{nmt1} in Figure 3.1 was excised as a *Bgl*II-*Eco*RI digest from the pREP1 plasmid. The 655 bp region in Figure 3.1 was amplified by PCR using a forward primer designed with a *Cla*I site (H1654) and a reverse primer designed with a *Xho*I site (H1655) for cloning into pKS-*ura4* (Figure 3.1) (see Table 3.2 for primer sequences used in this study). This amplified a region of genomic DNA (655 bp long) immediately downstream from the *pik1* coding sequence. This PCR fragment was cloned into pGEM-T Easy and sequenced for verification. For integration of the construct into the genome by homologous recombination, the construct was linearized

Figure 3.1: Schematic of the *pik1* allele replacement.

(A) The C-terminal end of the *pik1* cDNA (with and without mutations, C-*pik1*), in combination with the *nmt1* terminator sequence from the pREP1-*pik1* construct was cloned into the pKS-*ura4* plasmid with a *Bgl*II-*Eco*RI digest. Genomic DNA (655 bp) immediately downstream of the *pik1* genomic sequence was PCR amplified and cloned into the pKS-*ura4* plasmid after the *ura4* gene cassette with a *Cla*I-*Xho*I digest. The regions of homology are described in part B. The linearized *Bgl*II-*Xho*I fragment was used to transform diploids for homologous recombination. (B) As a result of homologous recombination, the *pik1* wild-type genomic sequence was replaced with the D709A, R838A and D709A, R838A mutations and the positive integrants were selected based on uracil prototrophy. Recombination occurred between homologous regions to produce the recombinant genomic locus.

A.



B.

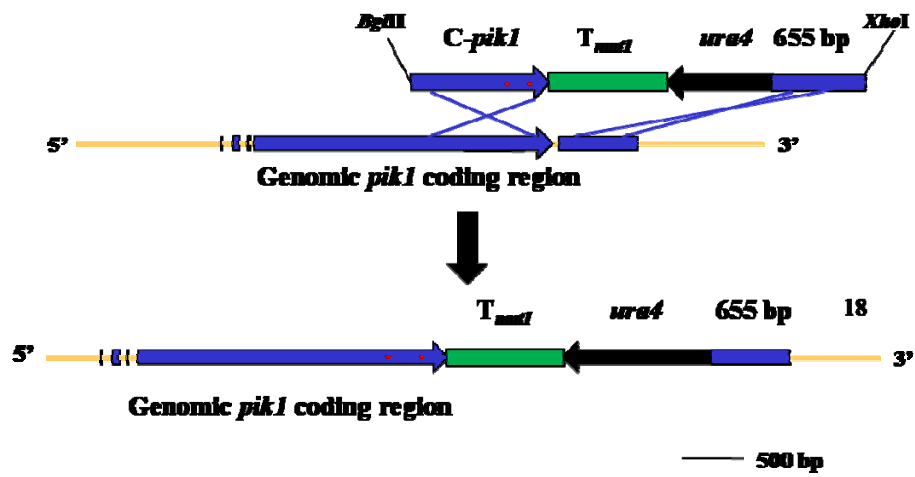


Table 3.2.

List of primers used in this study.

Primer Number	Sequence (5'-3')
H739	TAA ACA AAG TCA ATG AAG TAG
H1280	GTT CAA GGT TAC ATA TGC CAT CTT CG
H1284	GCA AGC CCA CAT GGT GGA TC
H1285	CTT GGA TCC CTA GTA AAT TCC GTT CGT AAT A
H1340	TAT ATC ATA CTT ACT GCA GTT AAA GGC TAG ACA TAA TGG TAA C
H1341	AAG GCC AAT TGT AGT GTT TGG ACG GCA CTT TAT GAT CTG TTT
H1653	ATG TCG ACG ATG GGA AAA TCA CAA TCA
H1564	AAC GGC CGC TCC TAA ACC AAT CCA TC
H1617	AGT CGA CTA ATG CCA TCT TCG AAT TCG
H1618	TAG ATC TTT ATT CTG CGC ATG ATA TGT C
H1629	AAG AGG TAT GAA AAT GAA TTC AAA AGG
H1654	ATC GAT TAT TGT ATT AAA ATC ACC ACA TTC
H1655	CTC GAG CAA AAG GTA CTT GTT TGA CTG ATA
H1662	TAA ACA AAG TCA ATG AAG TAG
H1679	TGC ATA CAT ATA GCC AGT GGG ATT TGT

with a *Bgl*II and *Xho*I double-digest (Figure 3.1). The fragment to be used for transformation was isolated after agarose gel electrophoresis. Agarose gel electrophoresis was performed in 0.75 to 1% agarose in TBE buffer (45 mM Tris-Borate; 1 mM EDTA). To visualize DNA fragments, the agarose gel included 0.5 µg/mL of ethidium bromide. The fragment of interest was cut out of the gel using a razor blade, and the DNA was isolated from the agarose gel using a Qiagen gel purification kit.

3.1.4.2. Allele replacement by homologous recombination

To obtain diploid cells for allele replacement by homologous recombination, matings of haploid cells were performed with the N2 h^+ and N3 h^- strains (Table 3.1) on ME solid medium for 24 hours at 25°C. The cross was then streaked onto EMM plates lacking adenine, the plates were sealed with parafilm and kept at 30°C for 4-6 days. Diploid cells were cultured in EMM lacking adenine at 30°C.

After 4-6 days of growth on EMM plates lacking adenine, a single colony of diploid cells was transferred to liquid EMM lacking adenine for 16 hours. The cells were transformed with ~10-20 µg of linearized plasmid DNA (Figure 3.1B) using a LiAc procedure (Section 3.1.3). After transformation, the cells were plated onto EMM plates lacking adenine and uracil to select for the positive integrants. After 4-5 days of growth at 30°C, colony PCR was used to identify and confirm positive integrants. Selective primer pairs (H739/H1662 and H1679/H1629) were used to identify the correct orientation of the inserted DNA in the genome. See Table 3.2 for the sequences of primers used.

3.1.4.3. Tetrad analysis

Tetrad analysis was used to establish the viability of cells carrying the integrated *pik1* alleles as described in Moreno *et al.* (1991). For the generation of the *pik1* mutant alleles, see Section 3.2.7. Briefly, the diploid cells heterozygous for the wild-type and mutant *pik1* insertions were incubated on ME plates at 25°C for 2 days to produce azygotic asci. Azygotic asci were isolated, and the four spores from each ascus were separated with a Zeiss tetrad micromanipulator and incubated on YES plates at 30°C for 5 days. My colleague Jae-Sook Park carried out the tetrad dissections. Restreaking the growing colonies on EMM lacking uracil was used to quickly assess for the presence of the integrated *ura4*⁺ gene cassette. Colony PCR, followed by sequencing, was performed to confirm the presence of the *pik1* mutations.

3.1.5. Colony formation assay

This assay was used to observe the effects of *pik1* wild-type and mutant ectopic expression on cell proliferation in *S. pombe* cells. *S. pombe* cells carrying the pREP plasmids were grown in liquid medium at 30°C (or 25°C for temperature-sensitive strains) to stationary phase ($\sim 10^8$ cells/mL). The cells were serially diluted 10-fold (from 10^7 to 10^2 cells/mL). An aliquot (5 μ L) of each dilution was spotted onto EMM lacking leucine plates containing or lacking 5 μ g/mL thiamine and supplemented with 5 mg/L phloxin B, which stains dead cells red. Spotting was performed by using a paper template which could be seen through the agar. The plates were incubated at 19°C, 25°C, 30°C and 37°C for 4-7 days, with growth recorded daily using a digital camera.

3.2. Molecular biology techniques

3.2.1. *E. coli* strains, media and culture conditions

E. coli was used for propagating all plasmid DNA. Three strains of *E. coli* were used in this study: XL1-Blue MRF', BL21(DE3) and CJ236 (see Table 3.3 for genotypes). *E. coli* were propagated in LB (10 g/L tryptone; 5 g/L yeast extract; 5 g/L NaCl) solid and liquid media supplemented with the appropriate antibiotics (ampicillin 100 µg/mL, kanamycin 30 µg/mL, tetracycline 10 µg/mL, chloramphenicol 25 µg/mL). *E. coli* cultures were generated by inoculating LB medium with a single colony grown on a plate at 37°C overnight. The liquid culture was allowed to grow in a shaking incubator for 16 hours at 37°C.

3.2.2. *E. coli* cell transformation

3.2.2.1. Heat-shock transformation

Chemically competent *E. coli* cells (see preparation below) were thawed on ice. A volume of 50 µL of chemically competent cells was then dispensed into a 1.5 mL eppendorf tube with ~200 ng to 1 µg of DNA and incubated on ice for 30 minutes. Cells were then heat-shocked at 42°C for 1 minute and immediately placed on ice for 2 minutes. LB supplemented with 25 mM glucose (from a 2 M stock) was then added to the heat-shocked cells and the mixture incubated at 37°C for 20 minutes. The cells were spread onto LB plates containing the appropriate antibiotic.

Table 3.3.List of *E. coli* strains used in this study

Strain	Genotype	Source
XLI-Blue MRF ⁺	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173$ <i>endA1 supE44 thi-1 recA1 gyrA96 relA1</i> <i>lac [F'proAB lacI^qZΔM15 Tn10 (Tet^r)]</i>	Stratagene
CJ236	<i>dut1, ung1, thi-1, relA1/pCJI05(F'cam^I)</i>	Takara
		Biomedicals
BL21(DE3)	<i>F⁻ ompT hsdS(rB⁻ mB⁻) dcm⁺ Tetr gal</i> <i>(DE3) endA Hte</i>	Novagen

3.2.2.1.1. Chemically competent *E. coli* cell preparation

Cells were made chemically competent using calcium chloride (CaCl₂). A single colony of *E. coli* cells was used to inoculate 50 mL of LB medium and the cells were allowed to grow overnight at 37°C with shaking. The next day, 4 mL of the starter culture was used to inoculate 400 mL of LB medium. Cells were grown at 37°C with shaking at 250 rpm to an apparent optical density of OD₅₉₀ 0.3-0.5 (Genesys 10, by Spectronic Unicam). After the cells reached the appropriate cell density, the culture flask was cooled on ice for 30 minutes and the cells centrifuged at 1,600 x g. The cell pellet was then resuspended in 10 mL of ice-cold 60 mM CaCl₂ solution and washed twice again in 60 mM CaCl₂ solution with the final wash incubated on ice for 30 minutes. The final cell pellet was resuspended in 2 mL ice-cold CaCl₂ solution, dispensed into pre-chilled 1.5 mL sterile polypropylene tubes and frozen immediately at -70°C.

3.2.2.2. Electroporation

Electrocompetent *E. coli* cells (see preparation below) were thawed on ice and 5 pg - 0.5 µg of plasmid DNA in 1 µL was added to an electroporation cuvette (2 mm gap) on ice. The electroporation apparatus (Bio-Rad, Gene Pulser) was set to 2.5 kV, 25µF and the pulse controller set to 200 ohms. After the electrocompetent cells had thawed, 50 µL of the cells were added to the 2 mm gap cuvette and tapped to mix the cells and DNA. The cuvette was then placed in the sample chamber and pulsed. SOC medium (0.5% yeast extract; 2% tryptone; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄; 20 mM glucose) was added to the cuvette at a volume of 1 mL. The

mixture was transferred to a 14 mL polypropylene round bottom tube and incubated for 30 to 60 minutes at 37°C in a shaking incubator. The cells were plated (100 µL – 900 µL) on solid LB medium containing the appropriate antibiotic.

3.2.2.2.1. Electrocompetent *E. coli* cell preparation

A single colony of *E. coli* cells was used to inoculate a 5 mL starter culture of LB medium and grown overnight in a shaking incubator at 37°C. A 500 mL culture was then inoculated with 2.5 mL of the starter culture and grown at 37°C with shaking at 300 rpm to an apparent optical density of OD₆₀₀ of ~0.5-0.7 (Genesys 10, by Spectronic Unicam). The flask with cells was chilled on ice for 30 minutes to 1 hour and transferred to three pre-chilled 250 mL bottles. The cells were centrifuged at 2,400 x g for 10 minutes at 2°C. The pellet was resuspended and washed with 40 mL ice-cold water two times, then resuspended in 40 mL 10% glycerol and centrifuged. Finally, the bacterial pellet was resuspended in 800 µL of 10% glycerol and 50 µL aliquots were dispensed into pre-chilled 1.5 mL polypropylene tubes and stored in -70°C.

3.2.3. Induction of protein expression in *E. coli* BL21(DE3) cells

The *pikI* gene was expressed in *E. coli* BL21(DE3) cells to analyze the lipid kinase activity of the protein. *E. coli* BL21(DE3) cells were transformed to ampicillin resistance with the pRSETB-*pikI* plasmid. A 2 mL starter culture inoculated with a single colony of these cells grown overnight at 37°C on an LB plate containing ampicillin, was prepared and allowed to grow overnight in a shaking incubator at 37°C. The next day, a 1/50 dilution of the starter culture in 25 mL was prepared and the cells

allowed to grow to an optical density OD₆₀₀ of 0.4-0.6 (Genesys 10, by Spectronic Unicam). This took approximately 4 to 5 hours. IPTG (isopropyl β -D-1 thiogalactopyranoside) was then added to the cells at a final concentration of 1 mM from a 100 mM IPTG stock. The cells were incubated at 37°C for 1 hour with vigorous shaking. After 1 hour, the cells were centrifuged at 2,060 x g for 10 minutes and resuspended in 3 mL 1 x PBS with protease inhibitors for use with bacterial cell extracts. The cells were then pressure homogenized using a 'mini' French pressure cell. The sample was run through the French press three times at 900 p.s.i. for immunoprecipitation. For western blot analysis, 1 mL of the 25 mL culture was used. The cells were frozen on dry ice and thawed at room temperature for 3 minutes, 3 times. Approximately 30 μ L of the homogenate was used for western blot analysis.

3.2.4. *E. coli* plasmid DNA preparation

An alkaline lysis method was used to prepare *E. coli* plasmid DNA. For mini-prep DNA, 3 mL of cells were cultured overnight at 37°C in a shaking incubator. Two mL of the cells were transferred to a 2 mL microcentrifuge tube, centrifuged for 5 minutes at top speed in a table top centrifuge, and the supernatant aspirated. The bacterial cells were resuspended in 100 μ L of solution 1 (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA). 200 μ L of alkaline lysis solution (0.2 N NaOH; 1% SDS) was added to the microcentrifuge tube and was mixed by gently inverting. The tube was kept on ice, and 150 μ L of ice-cold solution 3 (3 M potassium acetate in glacial acetic acid) was added. The tube was inverted several times and kept on ice for 5 minutes. The bacterial lysate was centrifuged at top speed in a bench top

microcentrifuge for 5 minutes and the supernatant transferred to a new 1.5 mL eppendorf tube. To precipitate the plasmid DNA, two volumes of 95-100% ethanol and 1/10 of 3 M sodium acetate were added. After inverting several times, the mixture was centrifuged at maximum speed for 10 minutes at room temperature. The supernatant was aspirated and the pellet washed with 750 μ L of 70% ethanol. After aspirating the supernatant, the pellet was allowed to air-dry at room temperature for 20 minutes. 50 μ L of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) was then added to dissolve the plasmid DNA pellet. For maxipreparation, a commercial Qiagen kit was used.

3.2.5. *S. pombe* RNA extraction

RNA was required as a template to generate the *pik1* and *ncs1* cDNA clones. A 200 mL culture of *S. pombe* cells were grown overnight to a cell density of 2×10^6 - 1×10^7 cells/mL. The cells were collected by centrifugation at $2,060 \times g$ for 15 minutes and homogenized by vortexing with acid-washed glass beads (0.45 - 0.5 mm diameter) and 4 mL of Trizol at a 4:1 (vol/vol) ratio. After adding 10 mL of Trizol to the sample, the contents were centrifuged to collect the glass beads and cell debris, and the top Trizol layer was pipetted to a fresh tube. The Trizol was extracted with chloroform, with 0.2 mL of chloroform added per 1 mL of Trizol. After centrifugation, the aqueous RNA-containing phase was collected, and the RNA was precipitated with isopropyl alcohol (0.5 mL isopropyl alcohol per 1 mL of Trizol). The sample was incubated at room temperature for 10 minutes and centrifuged at $12,000 \times g$ for 10 minutes at 4°C . After centrifugation, the translucent pellet was washed with 75% ethanol adding at least

1 mL of 75% ethanol per 1 mL of Trizol reagent initially used for the homogenization at 4°C. The RNA pellet was air dried, dissolved in RNase-free water and stored at -70°C.

3.2.6. Cloning of the *pik1* cDNA

The *pik1* cDNA was obtained using a first strand cDNA synthesis kit. RNA was extracted from log phase *S. pombe* cells as described in the section above. Reverse transcription was catalyzed by a Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase, which copies the cDNA from the RNA template using *NotI*-d(T)₁₈ primers. The cDNA was cloned by PCR amplifying the *pik1* cDNA from the first strand cDNA synthesis reaction using a forward primer that contained an *NdeI* site (H1280), and a reverse primer that contained a *BamHI* site (H1285) (see Section 3.2.9 for PCR conditions). The PCR product was purified by gel electrophoresis and incubated with the *NdeI* and *BamHI* enzymes. A ligation reaction was then prepared for cloning the *pik1* cDNA clones into the pRSETB and pREP vectors. The correctness of the PCR product was verified by sequencing.

3.2.7. Site-directed mutagenesis

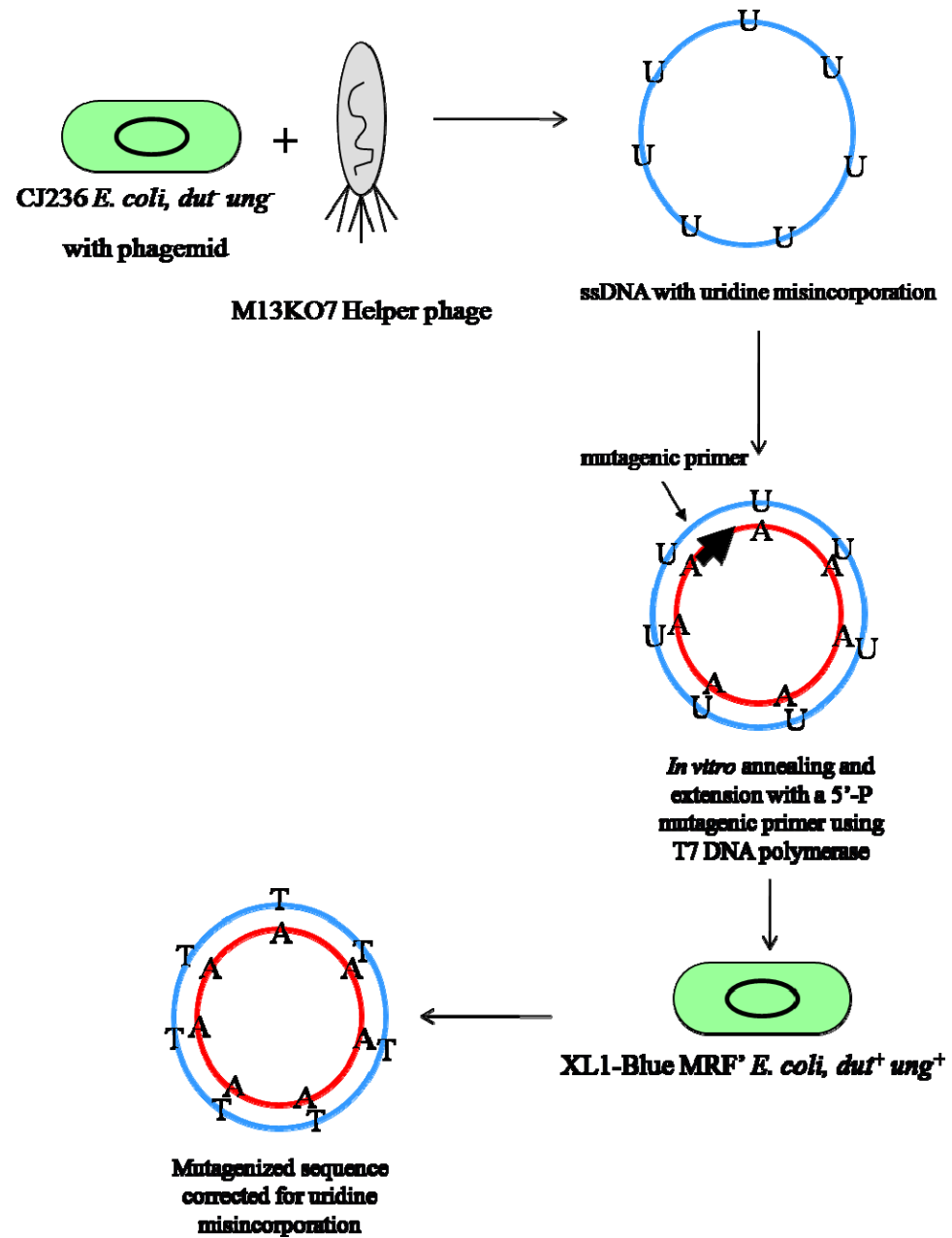
Mutagenic primers were used to introduce specific mutations into the *pik1* sequence. Mutagenesis was first performed to remove an internal *NdeI* site (bp 301 counting from the initiator methionine codon) in the *pik1* cDNA, using primer H1284. This mutation was silent with respect to the amino acid sequence. This was for ease of further cloning which required a *BamHI* and *NdeI* digest to move the entire sequence into the pREP vectors. The *pik1* sequence was then further modified by mutagenesis to

exchange an aspartic acid residue for alanine at residue 709 (using primer H1340), and to exchange an arginine residue for alanine at residue 838 (using primer H1341). The third double-mutant version of *pikI* carrying both the D709A and R838A mutations, was generated using recombinant DNA methods.

Site-directed mutagenesis was performed by the method of Kunkel *et al.* (1991). CJ236 *E. coli* cells were transformed with plasmid DNA carrying an F1 origin and the *pikI* sequence. The single stranded DNA (ssDNA) form of the plasmid was induced using helper bacteriophage. CJ236 cells are deficient in *dut* and *ung* which encode a dUTPase and uracil N-glycosylase, respectively. These deficiencies lead to the misincorporation of deoxyuridine in place of some thymidine residues (Figure 3.2). The transformed CJ236 cells were plated onto LB plates containing ampicillin and chloramphenicol to select for the introduced plasmid DNA and the CJ236 F factor. After inoculating 2 mL of LB liquid supplemented with ampicillin and chloramphenicol with cells from a single colony and incubating for approximately 7-8 hours in a 37°C shaking incubator, the F-pilus was allowed to regenerate by letting the culture stand at room temperature for 30 minutes. The cells were infected with M13K07 helper phage at a multiplicity of infection (MOI) of 10. After adding the required volume of M13K07 helper phage, the solution was gently mixed and left to stand at room temperature for 30 minutes to allow the phage to adsorb to and infect the cells. A larger 200 mL culture was then inoculated with this mixture and allowed to grow overnight in a 37°C shaking incubator. The next day the phage were precipitated and the ssDNA isolated. To separate the cells from the phage, the culture was centrifuged at 10,600 x g

Figure 3.2: Overview of the Kunkel method of site-directed mutagenesis.

In order to introduce a point mutation into a sequence, the wild-type sequence, in this case *pik1*, must be cloned into a plasmid that contains an F1 origin required for ssDNA production by bacteriophage. Once the CJ236 cells are infected, the supernatant containing the phage must be isolated to precipitate the phage which contains the ssDNA of the plasmid to be mutagenized. Once the ssDNA is isolated, an *in vitro* annealing and extension reaction with a single mutagenic primer is performed. The template strand, which contains misincorporated deoxyuridine, is corrected in the *dut*⁺ *ung*⁺ XL1-Blue MRF['] *E. coli*. DNA which contains the mutagenized sequence is then screened with restriction enzymes. The Kunkel method is advantageous because it greatly enhances the recovery of the replicative products of the strand that incorporates the mutagenic primer compared to other methods.



for 15 minutes. The supernatant, which contained the phage, was transferred to a clean bottle. The phage particles were precipitated by adding 1/4th volume of PEG solution (20% w/v PEG 8000; 2.5 M NaCl). The resulting mixture was incubated for 1 hour at 4°C and centrifuged at 10,600 x g for 15 minutes twice to remove all traces of the PEG solution in the supernatant. After the centrifugations, the pellet of phage particles was resuspended in 500 µL 1 x TE buffer pH 7.5 (10 mM Tris; 1 mM EDTA). The DNA of the suspended phage was extracted twice with 500 µL Tris-equilibrated phenol (pH 7.5-8.0), once with 500 µL phenol/chloroform/isoamyl alcohol (25:24:1), and then ethanol precipitated. Ethanol precipitation was carried out by adding 0.1 volume of 3 M sodium acetate (pH 5) and two volumes of ethanol, followed by an incubation at -20°C for 30 minutes or longer. The sample was centrifuged at 15,800 x g, washed with 700 µL 70% ethanol and recentrifuged. Finally, the sample was resuspended in 50-100 µL of 1 x TE buffer (pH 7.5). The sample was then quantified using a spectrophotometer (Genesys 10, by Spectronic Unicam) where an optical density OD₂₆₀ of 1 is equivalent to 36 µg/mL of ssDNA.

For each mutagenesis, a single mutagenic primer was used (Kunkel *et al.*, 1991). The primer was designed so that it would incorporate both the desired point mutation, and a translationally silent restriction enzyme site. The latter was introduced to facilitate the identification of clones carrying the introduced missence mutation. The H1340 primer introduced a *Pst*I site, while the H1341 primer introduced an *Mfe*I site. First, the primer was phosphorylated *in vitro* with T4 polynucleotide kinase (PNK) prior to use for mutagenesis. Four different primer: template molar ratios were used: 5:1, 10:1, 20:1 and 50:1. Each primer ratio was set up for a PNK reaction by adding 10x

PNK buffer, 0.4 mM ATP from a 100 mM stock, and 4 units of PNK in sterilized distilled water to 20 μ L /reaction. The reaction was allowed to proceed at 37°C for 1 hour and then terminated by incubating the reactions in a 65°C water bath for 10 minutes. The primers were then ready for annealing to the template. To each primer reaction, 1 μ L of ssDNA (~0.5 μ g – 1 μ g) and 1 μ L of 20x SSC buffer were added. The mixture was then mixed and centrifuged. The primers were allowed to anneal to the template DNA in a beaker of heated water (~70-90°C) which was allowed to cool to ~50°C. After the water had reached this temperature, the tubes were removed from the beaker and allowed to cool to room temperature for ~ 10 minutes. The tubes were centrifuged briefly (1 minute, 15,800 x g) to collect any condensation that had formed on the sides of the eppendorf tube. The primer and template mixture was then prepared for an *in vitro* DNA synthesis reaction with T7 DNA polymerase and T4 DNA ligase. In this reaction mixture, 0.4 mM ATP (from a 100 mM stock), 5x T7 DNA polymerase reaction buffer, 500 μ M dNTPs, double-distilled sterilized water and 1 unit each of T7 DNA polymerase and T4 DNA ligase were added. To stabilize the primer-template heteroduplex, each reaction was incubated on ice for 10 minutes. The reactions were incubated at 37°C for 1 hour and terminated by incubating each of the reactions at 65°C for 10 minutes. XL1-Blue MRF' *E. coli*, which are *dut*⁺ and *ung*⁺, were transformed to ampicillin resistance by adding 50 μ L of cells to a 2 mm gap cuvette and adding 3 μ L of the reaction mixture to the cells. The DNA was introduced into the cells by electroporation. The colonies were then screened for the presence of the mutated *pikI* sequence by mini-preparation of DNA, restriction enzyme digestion and agarose gel electrophoresis.

3.2.8. Plasmids for *pik1* and mutant allele expression in *S. pombe*

One of the approaches used in this study was to ectopically express *pik1* under the control of the *nmtI* promoter (Figure 3.3). This was performed by cloning the *pik1* sequence into the pREP (1/41/81) plasmids (Maundrell, 1990; Maundrell, 1993; Basi *et al.*, 1993). The pREP 1/41/81 vectors differ in their *nmtI* promoter sequence, which results in different levels of expression with pREP1 producing the highest level of expression and pREP81 producing the lowest level of expression. The *pik1* cDNA contained an internal *NdeI* site, thus, the initial *pik1* sequence was cloned in two steps: first the larger ~2.2 Kb *NdeI*-*Bam*HI fragment of the *pik1* cDNA was cloned, and second, the smaller ~300 bp *NdeI*-*NdeI* fragment of the *pik1* cDNA was cloned. For further ease of cloning, the internal *NdeI* site was mutagenized using the Kunkel method in pRSETB. This introduced a silent C to T mutation at nucleotide 301. The full-length *pik1* cDNA was then cloned into the pREP1, pREP41 and pREP81 thiamine repressible *S. pombe* expression vectors using an *NdeI*-*Bam*HI digest (Figure 3.3).

3.2.9. PCR amplification

Routine PCR: 100 µL PCR reactions were set up. 10 ng of template DNA was used and 10 pmoles of primer DNA from a stock of 100 pmole/µL was added. PCR reaction buffer was supplied by the manufacturer and was added to 1x from a 10x stock. When *Taq* polymerase was used, 50 mM MgCl₂ was added to the mixture. 2 µL of a 10 mM mixture of dNTPs (to a final concentration of 0.2 mM) was then added and finally 1 µL of *Taq* polymerase (5 units/µL) was added to the reaction

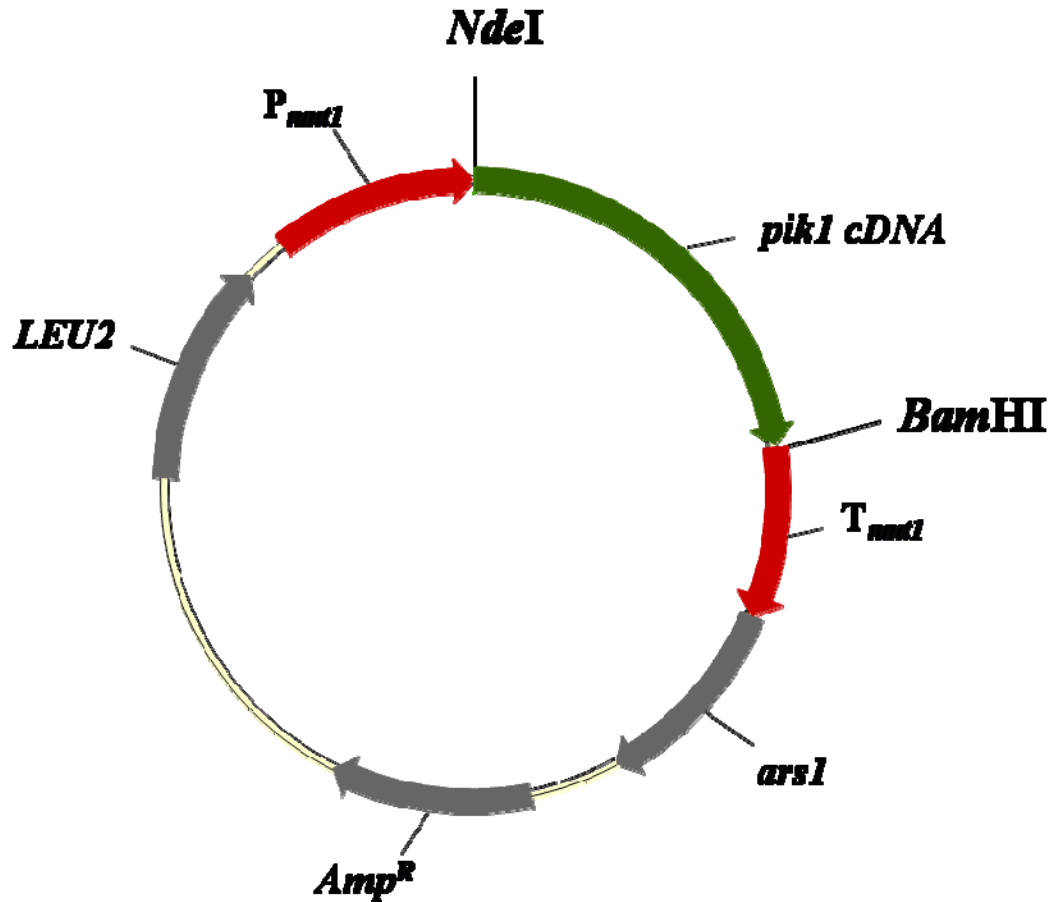


Figure 3.3: Schematic representation of the pREP vectors with *pik1* alleles.

The pREP vectors (pREP1/41/81) have a thiamine repressible promoter P_{nmt1} , that is repressed when cells are grown in the presence of 5 $\mu\text{g/mL}$ of thiamine. The plasmid also contains a terminator sequence (T_{nmt1}) and an *ars* sequence for the autonomous replication of the plasmid in *S. pombe* cells. The *S. pombe* pREP vectors are also shuttle vectors that can be propagated in *E. coli* and thus, have an ampicillin resistance gene (Amp^R) for that purpose.

mixture. Samples were melted at 95°C initially for 5 minutes and when cycling, for 1 minute. An annealing temperature of 55°C for 1 minute was initially used and altered as needed. Extension time was based on the template size with 1 minute of extension time per kb of template at 72°C. A final extension cycle was added for 5 minutes at 72°C. The PCR reaction was usually allowed to cycle 30 times.

Colony PCR: 50 µL PCR reactions were set up. 25 µL of 20 mM NaOH was added to 1.5 mL polypropylene tubes. With a sterile pipette tip, a little bit of the colony to be screened was transferred to the tube. The sample was boiled for 15 minutes, vortexed and centrifuged for 1 minute at room temperature at 15,800 x g. An aliquot (1 µL) of the solution was transferred to a 50 µL PCR reaction and allowed to cycle for 40 cycles to obtain sufficient PCR product. The PCR reactions were run on a 0.8% agarose gel containing ethidium bromide and the product visualized under UV light. For sequencing, the PCR product was cloned into the TA cloning vector pGEM-T Easy and sequenced with a model 370A automated sequencer.

3.2.10. *S. pombe* protein extraction and estimation

Yeast proteins were extracted using the ‘mini’ French pressure cell. The *S. pombe* cells were pressure homogenized in either 1 x PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄) or PtdIns 4-kinase buffer (25 mM HEPES, pH 7.4; 10 mM MgCl₂) with protease inhibitors for yeast according to the manufacturer (see list of Materials and Supplies) at 900 p.s.i., 3 times at 4°C. Protein concentrations were estimated by the method of Bradford (Bradford, 1976) using the Bradford reagent which is a protein dye (Brilliant blue G in phosphoric acid and methanol) that causes a

shift in dye absorption from 465 to 595 nm upon binding to protein. A standard curve was prepared using diluted bovine serum albumin (BSA) (0.1 mg/mL from a 10 mg/mL stock). BSA was diluted to a range of 0.5 µg to 5 µg in the appropriate buffer (in a volume of 500 µL). An equal amount (500 µL) of Bradford reagent was then added to the BSA and the apparent optical density OD₅₉₅ measured (Genesys 10, by Spectronic Unicam). The protein sample of unknown concentration was quantified at the same time. Aliquots of 5-10 µL of total protein were diluted in 500 µL of the appropriate buffer, and absorbance read. A standard curve was produced and the total protein content of the sample estimated based on linear regression analysis.

3.2.11. SDS-polyacrylamide gel electrophoresis and western blotting for Pik1p

SDS-polyacrylamide gel electrophoresis was performed using the Bio-Rad mini-PROTEAN 2 system. To detect Pik1p protein levels, a 7.5% SDS polyacrylamide gel solution was prepared. The w/w ratio of bisacrylamide: acrylamide used was 1:37.5. Resolving gels were made by mixing the 30% acrylamide solution (2.5 mL), double-distilled sterilized water (3.6 mL), 1.0 M Tris pH 8.8 (3.75 mL), 20% SDS (50 µL), 10% ammonium persulfate (100 µL) and TEMED (N,N,N',N'-tetramethylethylenediamine) (10µL). Stacking gels were made at 5% acrylamide. 200 µL of the protein sample was added to 50 µL of 5x SDS loading dye (250 mM Tris-HCl, pH 6.8; 10% w/v SDS; 0.5% w/v bromophenol blue; 50% v/v glycerol; 12.5% β-mercaptoethanol added just before use) and boiled for 3 minutes before loading onto the gel. 30 µL of the cell homogenate with SDS loading dye was loaded. Samples were run at 200 V for 40 minutes.

Once the gel had completed running, the gel was placed over a pure nitrocellulose membrane of pore size 0.45 μm . The gel, after being placed between two fiber pads and filter papers, was placed in the transfer apparatus (Bio-Rad Mini Trans-Blot electrophoretic transfer kit) in transfer buffer (25 mM Tris; 192 mM glycine; 20% v/v methanol) for wet transfer. The transfer was conducted at 100 V for 1 hour. The membrane was then incubated in 2% skim milk (in powder form, w/v) in 1 x PBS overnight at 4°C. The next day, the membrane was incubated with a 1:1000 dilution of primary rabbit IgG antibody against Pik1p (Desautels *et al.*, 2001) in 2% skim milk for 1 hour at room temperature, on a rotating platform at a gentle speed. The membrane was then washed 3 times with 1 x PBS and 1x PBST (1 x PBS; 0.1% vol/vol Tween 20) alternating the washes with rigorous hand shaking. Next, the membrane was incubated with a 1:5000 dilution of goat anti-rabbit IgG-HRP (horse-radish peroxidase) secondary antibody in 2% skim milk for 1 hour with gentle shaking. After the incubation, the membrane was washed 3 times with 1 x PBS and 1 x PBST alternating the washes with vigorous shaking. To visualize the protein, the membrane was incubated for 5 minutes at room temperature with a mixture of solutions 'A' and 'B' (40:1) from the ECL HRP chemiluminescent detection kit. The membrane was then wrapped in plastic wrap and exposed to film for 1 minute, 5 minutes and 10 minutes. The film was developed using a commercial X-ray film developer.

3.2.12. Immunoprecipitation of Pik1p from *E. coli* BL21(DE3) and *S. pombe* cell homogenates

The Pik1p protein was synthesized in BL21(DE3) cells and the cells pressure homogenized according to Section 3.2.10. *S. pombe* cell lysates were prepared according to Section 3.2.10 in 1 x PBS buffer with protease inhibitors for yeast. One mL of the lysate was used for immunoprecipiation. BSA and Tween 20 were both added to the 1 mL of lysate at 0.5% (w/v and v/v, respectively) and the lysate centrifuged at 2,060 x g for 30 minutes. The supernatant was retrieved and pre-treated with protein A-sepharose beads by adding 10 μ L of beads for every 200 μ L of supernatant. The protein A-sepharose beads were previously hydrated in buffer (0.2 M NaH_2PO_4 ; 0.15 M NaCl) for 30 minutes or longer at room temperature on a rotating wheel. One gram of powder swells to 3 to 4 mL of hydrated gel. For long term storage, the beads were stored in buffer (see above) with 0.1% sodium azide. The supernatant was treated with the protein A-sepharose beads for 1 hour at 4°C on an orbital shaker. The mixture was then centrifuged at 2,060 x g for 10 minutes at 4°C. 200 μ L of the supernatant was taken, and BSA added to the mixture to 0.5% (w/v). A 1:40 dilution of α -Pik1p antiserum was then added. The mixture was allowed to incubate for 1 hour at room temperature on a rotating wheel. 50 μ L of protein A-sepharose beads were added and the mixture incubated at room temperature for 1 hour on a rotating wheel. After the incubation, the mixture was centrifuged at 2,060 x g for 5 minutes at 4°C and the supernatant was aspirated into waste. The beads were washed with 500 μ L of 1 x PBS with gentle vortexing, centrifuged at 2,060 x g for 5 minutes and the supernatant aspirated into waste. The beads were washed again with 500 μ L of wash 2 solution (100 mM Tris-HCl, pH 7.4; 50 mM LiCl), gently vortexed, centrifuged as described previously and the supernatant aspirated into waste. The beads were washed finally

with 500 μ L of wash 3 solution (25 mM HEPES, pH 7.4; 100 mM NaCl; 1 mM EDTA) with gentle vortexing, centrifuged as described previously and the supernatant aspirated into waste. The beads were then used for lipid kinase activity assays as described in Section 3.2.14 in 50 μ L of PtdIns 4-kinase buffer.

3.2.13. Protein-protein interaction studies

3.2.13.1. ELISA

A 50 mL culture of *S. pombe* cells was inoculated at an initial cell density of 1×10^5 cells/mL. The cells, which carried pREP1 plasmids for expression of wild-type and mutant *pik1* alleles, were incubated for 24 hours at 30°C in the presence or absence of thiamine. Cell extracts were prepared using a French press ‘mini’ cell (3 passages at 900 p.s.i.) in 1 x PBS with protease inhibitors for yeast at 4°C. Multiwell (96) plates were coated with 10 μ g/mL of purified Cdc4p protein (Slupsky *et al.*, 2001) by incubating the plate in a humidified container overnight at 30°C. The plates were then blocked by incubating the wells with 2% (w/v) powdered skim milk in 1 x PBS at room temperature for 1 hour. Serial two-fold dilutions of cell lysates (from 56 μ g to 14 μ g of protein) were added to the Cdc4p-coated multiwell plates and incubated ~16 hours at 4°C. The wells were washed 4 times with 1 x PBS-0.01% v/v Tween 20 and incubated with a 1:1000 dilution of polyclonal rabbit anti-Pik1p serum (Desautels *et al.*, 2001) for 4 hours at room temperature. After washing, the plates were incubated for 1 hour at room temperature with goat anti-rabbit IgG-HRP at a dilution of 1:5000. The plates were developed with TMB (3,3',5,5'-tetramethylbenzidine) solution (100 mM NaOAc, pH 6.0 with 1 mg/mL of TMB supplemented with 4 μ L of H₂O₂ per 10 mL solution just

before use) for 10 minutes and the reaction stopped with H₂SO₄ (final concentration of 0.5 N). Changes in optical density were estimated with a Molecular Devices microplate reader at wavelengths 650 nm and 450 nm, and the data analyzed using Softmax Software, version 2.34.

3.2.13.2. Yeast two-hybrid assays

Yeast two-hybrid protein-protein interaction studies were performed as described in Kohalmi *et al.* (1997) utilizing the leucine selectable pBI880 vector carrying the Gal4p-DBD (DNA binding domain) fused to *cdc4* and *ncs1* gene sequences (see next Section) and the tryptophan selectable pBI771 vector carrying the Gal4p-TA (transactivating domain) fused to the N-terminal end of the C-terminal region (residues 507 to 851) of the wild-type and mutant *pik1* alleles (see next Section). Vectors were also constructed which contained the full-length *pik1* coding sequence in pBI771. Both the pBI880 and pBI771 vector constructs were introduced into *S. cerevisiae* YPB2 cells one after the other using a lithium acetate procedure (TRAFO). After the second transformation, 500 µL of 1 mL of transformed cells were plated on synthetic dextrose (SD) medium lacking leucine and tryptophan to select and quantify clones that carried both plasmids. The other 500 µL of 1 mL of transformed cells were plated onto SD medium lacking leucine, tryptophan, histidine and supplemented with 3- amino- 1', 2', 4'- triazole (3-AT) to determine which cells scored positive for the presence of protein-protein interactions. 3-AT was added to a final concentration of 5 mM from a 2 M stock. Upon a positive interaction, the Gal4p DBD and TA domain reconstitute the Gal4p transcription factor resulting in the expression of histidine prototrophy and the

lacZ gene. Thus, cells in which an interaction occurs are able to grow on medium lacking histidine and containing 3-AT, and turn blue in the presence of X-gal substrate, respectively. Cells were grown at 30°C for 5 days on SD –Leu –Trp plates and 7 to 9 days on SD –Leu –Trp –His + 3-AT plates.

3.2.13.2.1. Plasmids for yeast two-hybrid

The ‘bait’ and ‘prey’ vectors used were the pBI880 and pBI771 vectors, respectively (Kohalmi *et al.*, 1997) (Figure 3.4 A and B). The C-terminal domain of Pik1p (507-851) in pBI771 was obtained from Michel Desautels as a *SalI-NotI* digest (Desautels *et al.*, 2001). The full-length *cdc4* cDNA was cloned into the pBI880 vector previously as well (Desautels *et al.*, 2001). In order to create the pBI771 C-terminal domain constructs of Pik1p carrying the D709A, R838A and D709A, R838A mutations, a portion of the *pik1* sequence from pBI771 was cloned into pBluescript KS(-) as a *SpeI-SalI* digest. This construct was then used as a template for mutagenesis using the Kunkel method (see Section 3.2.7). Once the appropriate mutations were introduced, they were cloned back into the pBI771 *pik1* 508-851 sequence by a *BglII-SpeI* digest. To create the pBI771 Pik1p full-length (1-851) construct, both PCR and subcloning were used, respectively, first by cloning the N-terminal region of *pik1*. To clone the N-terminal region of Pik1p, PCR was employed to amplify the region from the START codon to the *BglII* site in the middle of the *pik1* sequence, using pREP1-*pik1* as a template. The PCR primers were designed so that the forward primer carried a *SalI* site (primer H1617) which would allow cloning of the N-terminal domain of Pik1p in-frame with the Gal4p TA domain. The reverse primer included the *BglII* site (primer

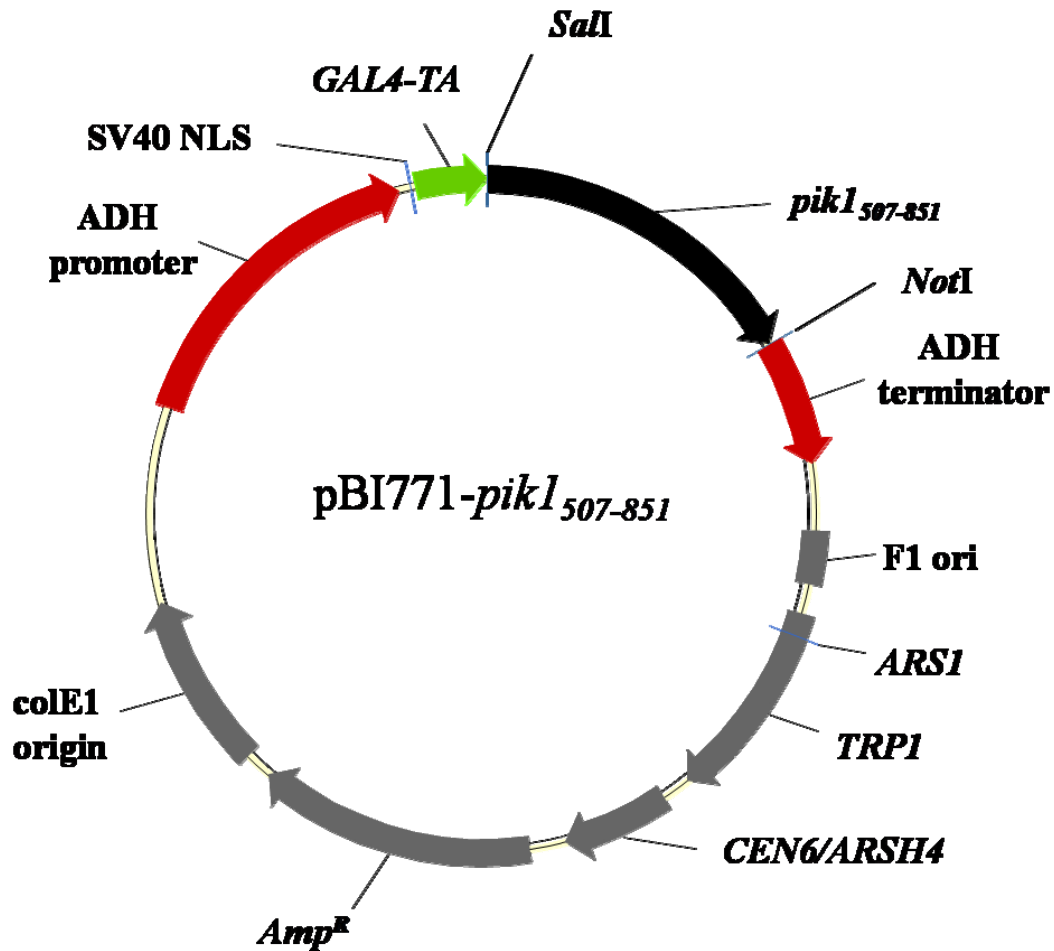


Figure 3.4: Yeast two-hybrid vectors.

The yeast two-hybrid vectors were used for examining the protein-protein interaction between the C-terminal region of *pik1* and Cdc4p in *S. cerevisiae*. (A) The pBI771 ‘prey’ vector. The *pik1* cDNA, residues 507-851, were cloned into pBI771 as a fusion with the Gal4p transactivating (TA) domain. The plasmid sequence is also equipped with sequences required for its propagation in *E. coli* and *S. cerevisiae*. For selection in *S. cerevisiae*, the tryptophan selectable marker was cloned into this vector. (B) The pBI880 ‘bait’ vector. Both the *cdc4* and *ncs1* cDNAs were cloned into the pBI880 vector fused to the Gal4p DNA binding domain (DBD). The plasmid sequence is also equipped with sequences required for its propagation in *E. coli* and *S. cerevisiae*. For selection in *S. cerevisiae*, the leucine selectable marker was cloned into this vector.

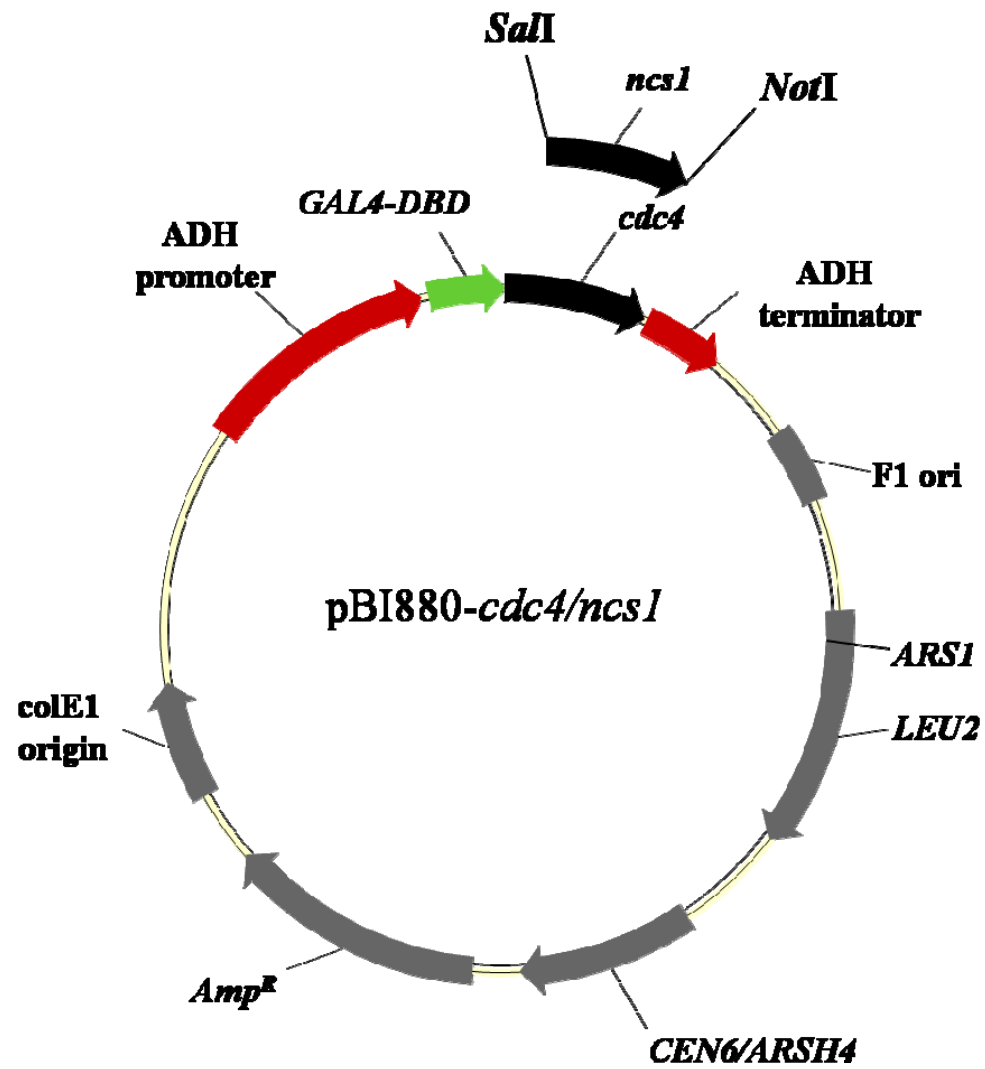


Figure 3.4 B continued.

H1618). The PCR fragment, which was generated using *Taq* polymerase, was cloned into the TA cloning vector pGEM-T Easy to acquire sufficient amounts of insert for cloning and sequencing. After verifying that the sequence was correct, the N-terminal region of *pik1* was then cloned into the pBI771-*C-pik1* construct to generate the full-length *pik1* sequence using a *SalI* single digest.

To create the pBI880 *ncs1* construct, PCR of the first strand cDNA synthesis reaction was employed (see Section 3.2.6). The forward primer was designed to contain a *SalI* site to clone the *ncs1* coding sequence in-frame with the Gal4p DBD (primer H1563). The reverse primer was designed with a *NotI* site for cloning into pBI880 (primer H1564). The PCR product was cloned into the TA cloning vector, pGEM-T Easy, to obtain sufficient amounts of insert and for sequencing.

3.2.14. Lipid kinase assays

The following assay is a modified version of the assay described in Mahon *et al.*, 2005. Cultures of *S. pombe* cells carrying the episomes for expression of *pik1* and the mutant alleles were incubated for 24 hours in the presence and absence of thiamine at 30°C, or 25°C as appropriate. Cells were harvested by centrifugation (2,000 x *g* for 5 minutes) and resuspended in lipid kinase buffer (25 mM HEPES, pH 7.4; 10 mM MgCl₂). Cell lysates were prepared by running the cell suspensions through a ‘mini’ French pressure cell 3 times at 900 p.s.i.. The protein content from each cell lysate preparation was quantified using the Bradford reagent and diluted to 0.05 µg – 1.2 µg per 50 µL reaction (Bradford, 1976). Each diluted cell extract was incubated with 10 µCi [γ- ³²P] ATP for 15 minutes at room temperature and the reactions stopped with 6

M HCl to a final concentration of 1.7 M. Lipids were extracted with three volumes of chloroform: methanol (1:1, vol/vol), vortexed for 10 seconds and centrifuged for 5 minutes. The organic layer containing the labeled lipid products was removed and dispensed into a fresh 1.5 mL eppendorf tube. The lipids were further extracted with a half volume of methanol: 1 N HCl (1:1, vol/vol), vortexed and centrifuged as previously described. The organic phase was again retrieved and dispensed into a fresh tube which was then dried under N₂ gas. The lipids were dissolved in 4 µL of chloroform: methanol (1:1, vol/vol) and spotted onto a Silica gel 60 thin layer chromatography (TLC) plate which had been baked previously for 30 minutes at 100°C. The spotted TLC plates were placed in a chromatography chamber equilibrated previously for two hours in 75 mL of freshly made developing solution (1-propanol: 2 M acetic acid (13.7:7)). The plates were run for 6-8 hours and dried overnight in a fume hood. Plates were then either exposed to Kodak BioMax XAR film for 2-3 days or scanned under 10% methane in argon using the BIOSCAN AR-2000 imaging scanner for radio-TLC. The plates were scanned for 30 seconds. After scanning, the WinSCAN 2D software version 1.05 was used to visualize the radioactivity on the plates. The silica spots carrying the radiolabelled lipids were then scraped off the plates and added to 4.5 mL of Aquasol, and the radioactivity measured using a liquid scintillation counter. Data were then corrected for counting efficiency and decay and expressed as disintegrations per minute (DPM). To calculate the DPMs, background values were measured by scraping 3 non-radioactive spots on the TLC plate and obtaining the counts per minute (CPM) of these spots by liquid scintillation counting. An average value of the 3 spots was calculated. This “background” value was subtracted from the counts per minute (CPM)

of the lipid spot. This value was then corrected for decay by determining the percentage of activity remaining of the ^{32}P - γ -ATP and the efficiency of detection of ^{32}P decay by the liquid scintillation counter (99%).

To determine if Cdc4p purified protein affected *pik1* lipid kinase activity, the effects of increasing the molar concentration of Cdc4p on cell homogenates carrying expressed Pik1p protein were assessed. Here, 0.4 μg of homogenates of cells in which the *pik1*^{wt} allele had been ectopically expressed was incubated with 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM and 10 mM of purified Cdc4p protein in a 50 μL reaction. Alternatively, the effects of purified Cdc4p on *pik1* lipid kinase activity was tested by incubating varying quantities of total cell homogenates (0.01-2.4 μg of total protein) with 1 mM of purified Cdc4p in a 50 μL reaction.

The nature of the lipid spots on the TLC plates were identified by reference to lipid standards purchased from Avanti Polar Lipids. 50 μg each of PtdIns, PtdIns4P, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ were run in parallel and visualized using iodine vapour by placing the plates and iodine crystals in a chromatography chamber. The plates were allowed to develop for 1 hour.

3.3. Microscopy

3.3.1. Fixation of yeast cells

3.3.1.1. Paraformaldehyde fixation

A 17.5% formaldehyde solution (5x) was prepared (Moreno *et al.*, 1991). Paraformaldehyde (8.75 g) was weighed and 1 x PBS was added to 50 mL. One mL of 1 M NaOH was then added and the solution incubated at 65°C for 20 minutes. The

paraformaldehyde was dissolved by shaking and then centrifuged at 2,060 x *g* for 30 minutes. The clear supernatant was used to fix the cells by adding 5 mL to 20 mL of culture. The cells were fixed by incubating the mixture on a rotating platform for 30 minutes. The cells were then centrifuged at 2,060 x *g* for 5 minutes and washed 3 times with 1 x PBS. The cells were stored at 4°C in 1 x PBS containing 1 mM NaN₃. Cells were kept at 4°C for a maximum of 1 month prior to examination and then discarded.

3.3.1.2. Methanol fixation

Methanol fixation of *S. pombe* cells was as described in Moreno *et al.* (1991). A 20 to 50 mL culture of cells at the mid-logarithmic phase of growth was collected using a vacuum driven 0.22 µm disposable bottle top filter. The cells were washed once with 100% ice-cold methanol. The cells were then incubated in 30 mL of ice-cold methanol in the bottle top filter. The apparatus with the cells was incubated at -20°C for 10-15 minutes. The flaked-off cells were scraped from the filter membrane, transferred to a 50 mL polypropylene Falcon tube and centrifuged at 2,060 x *g* for 5 minutes. The cells were rehydrated by washing sequentially in 75%, 50%, 25% and 0% methanol in 1 x PBS. Cells were stored at 4°C in 1 x PBS supplemented with 1 mM NaN₃.

3.3.2. Bright field microscopy and cell length measurement

Cells that were formaldehyde fixed were visualized under a bright field objective. An Olympus 1x70 inverted microscope with a 60x 1.4 NA Plan-apo objective, appropriate filter sets and an RT-Slider (SPOT) CCD camera (Carsen Scientific Imaging Group, Markham Canada) was used for this purpose. The images

were digitally recorded and analyzed using SPOT advanced software. The images (600x magnification) were then printed and cell length measurements taken with a ruler. Cell length was estimated relative to a micrometer calibration bar. Between 50-200 cells were measured for each experiment and the results expressed as a relative frequency distribution.

3.3.3. Fluorescence microscopy

3.3.3.1. Visualization of F-actin structures with FITC-conjugated phalloidin

An aliquot (20 μ L) of formaldehyde fixed cells were incubated with 5 μ L of FITC-conjugated phalloidin (0.2 mg/mL in 1 x PBS with 1% Triton-X) and incubated for 30 minutes in the dark. To achieve this, the 1.5 mL polypropylene tubes were wrapped in tin foil. One mL of 1 x PBS was then added to each tube followed by centrifugation at 4,000 x g for 1 minute. The supernatant was removed by suction and the cells resuspended in 50-100 μ L of 1 x PBS. The cells (20 μ L) were then placed on poly-L-lysine (1 mg/mL) coated coverslips for 5 minutes. Excess liquid was removed from the coverslip with a p20 pipetteman and the coverslip allowed to air dry. The coverslip was then placed onto a glass slide containing 12 μ L of mounting solution (1 mg/mL *p*-phenylenediamine; 50% glycerol) containing or lacking 1 μ g/mL DAPI (4',6'-diamidino-2-phenylindole dihydrochloride). DAPI stains DNA. The coverslip was sealed with clear nail polish.

3.3.3.2. Visualization of the nucleus and septum with DAPI and calcofluor white

An aliquot (50 μ L) of formaldehyde fixed cells were added to 450 μ L of 1 x PBS and 2.5 μ L of a 10 mg/mL stock solution of calcofluor white in distilled and sterilized water was added. The cells were incubated at room temperature on a rotating wheel for 15 minutes. The cells were then washed 5 times in 1 x PBS and the final pellet resuspended in 500 μ L to 1 mL of 1 x PBS. An aliquot (20 μ L) of cells were added to a poly-L-lysine coated coverslip for 5 minutes and the excess removed with a p20 pipetteman. The coverslip, after being air dried to remove excess moisture, was then placed on 12 μ L of mounting solution containing DAPI on a glass slide. The coverslip was sealed with clear nail polish.

3.3.3.3. Visualization of Myo2p and Pik1p by indirect immunofluorescence microscopy

To visualize intracellular molecules and structures by indirect immunofluorescence, the cell walls of the methanol fixed cells were partially digested (Moreno *et al.*, 1991). First, 100 μ L of a methanol fixed cell suspension was added to 900 μ L of solution (1 x PBS; 1.2 M w/v sorbitol) and the cells collected by centrifugation at 13,000 x g. The methanol fixed cells were digested with 0.25 mg/mL Zymolase 20T and 0.25 mg/mL lysing enzyme in 1 mL PBS buffer containing 1.2 M sorbitol. The cells were incubated for 15 minutes and an equivalent volume of 1 x PBS with 2% Triton-X was immediately added. The cells were collected by centrifugation at 15,800 x g for 2 minutes and then washed 3 times with 1 x PBS. One mL of PBAL (1 x PBS; 100 mM lysine-HCl; 1% fatty acid-free BSA) was added to the final cell pellet and incubated on a rotating wheel for 30 minutes. Finally, the cells were resuspended

in 500 μ L of PBAL, 1:100 dilution of primary antibody or pre-immune serum added and the mixture incubated overnight at room temperature on a rotating wheel. The Pik1p antibodies were available in the lab (Desautels *et al.*, 2001). The Myo2p antibody was a generous gift from Dr. M. Balasubramanian (University of Singapore). The Pik1p and Myo2p antibodies were from rabbits.

After the overnight incubation, cells were washed 5 times with 1 mL PBAL with the final wash consisting of a 20 minute incubation on a rotating wheel. A 1:100 dilution of a secondary antibody in PBAL was added. This was a goat anti-rabbit IgG-Texas Red conjugate. The solution was incubated for 1 hour in the dark at room temperature on a rotating wheel. The cells were then washed 5 times with 1 x PBS with the final wash being incubated for 20 minutes on a rotating wheel. The cells were resuspended in 100-500 μ L of 1 x PBS and mounted onto glass slides with mounting solution lacking DAPI as previously described.

An Olympus 1x70 inverted microscope with a 60x 1.4 NA Plan-apo objective, appropriate filter sets and an RT-Slider (SPOT) CCD camera (Carsen Scientific Imaging Group, Markham Canada) was used with the whole image processed for brightness and contrast using Adobe Photoshop 6.0.

3.4. Protein alignments and phylogenetic trees

Protein alignments were performed using ClustalW. Phylogenetic trees were made from a ClustalW alignment of the kinase domains of each of the proteins listed in Table 3.4. The kinase domains were determined according to Pfam. Phylogenetic analysis was performed by programs in the PHYLIP software package. Specifically,

alignments were sampled for bootstrap analysis by Seqboot and distances were calculated with the PAM option of Protdist (for peptide sequences). Consensus trees were calculated by Consense, and branch lengths were superimposed on consensus trees by Fitch. Completed trees were viewed by TreeView and manipulated for presentation in Microsoft Powerpoint.

Table 3.4.

Sources for kinase domain amino acid sequences used for phylogenetic analysis of lipid

kinases

Protein	Accession number/Locus	Kinase domain (residues from Pfam)
<i>S. pombe</i> Pik1p	NP_594842	578-799
<i>S. cerevisiae</i> Pik1p	CAA63231	791-1041
Bovine PtdIns4KIII β	002810	559-816
Human PtdIns4KIII β	BAA21661	559-816
<i>A. thaliana</i> PtdIns4KIII β	CAB37928	857-1069
<i>S. pombe</i> Stt4p	SPBC577.06	1618-1825
<i>S. cerevisiae</i> Stt4p	NP_013408	1641-1848
<i>S. pombe</i> Lsb6p	CAB52282	154-505
<i>S. cerevisiae</i> Lsb6p	NP_012435	170-507
<i>S. pombe</i> Fab1p	CAA17054	1683-1916
<i>S. cerevisiae</i> Fab1p	BAA09258	2029-2266
<i>S. pombe</i> Pik3p	SPAC458.05	541-799
<i>S. cerevisiae</i> Vps34p	YLR240W	619-873
<i>S. pombe</i> Tor1p	SPBC30D10.10c	1981-2335
<i>S. cerevisiae</i> Tor1p	YJR066W	2119-2470
<i>S. pombe</i> Tor2p	SPB216.07c	1984-2337
<i>S. cerevisiae</i> Tor2p	YKL203C	2123-2474

Chapter 4: Results

4.1. Introduction

The existence of a physiologically relevant interaction between the products of the *S. pombe* *cdc4* and *pik1* genes has been hypothesized based on evidence from yeast two-hybrid assays and ELISAs. These experiments demonstrated that Cdc4p interacted with the C-terminal half of a putative phosphatidylinositol 4-kinase, Pik1p (Desautels *et al.*, 2001). In that study, *cdc4* was shown to interact with *pik1* in an allele specific manner such that *cdc4*^{F12L}, *cdc4*^{G19E}, *cdc4*^{R33K}, *cdc4*^{F79S}, and *cdc4*^{G82D}, interacted with *pik1*, but *cdc4*^{G107S} did not. No direct evidence for a physiological interaction between these two proteins in *S. pombe* was presented however, and an interaction with the full-length Pik1p protein was not established. I addressed these two issues in my thesis. The major approaches taken were to investigate the importance of Pik1p lipid kinase activity and of the Pik1p-Cdc4p interaction in cytokinesis. To this end, the first step was to attempt to introduce two mutations in Pik1p: one to abolish lipid kinase activity and the other to abolish Cdc4p-binding activity. The ectopic expression of the wild-

type and mutant *pik1* alleles, as well as the integration of these mutations into the genome, were then used to explore *pik1* function *in vivo*.

4.2. The *S. pombe* ectopic expression system

A *pik1* cDNA clone was obtained by reverse transcription PCR, using gene specific primers and a total RNA preparation as template, and this cDNA clone was transferred into a series of pREP vectors. The cloned sequence included an internal *NdeI* restriction enzyme site which made subcloning tedious. The internal *NdeI* site was removed by site-directed mutagenesis by introducing a silent C to T mutation at nucleotide 301 starting from the ATG start codon of the sequence. Compared to the published genomic DNA sequence (Wood *et al.*, 2002), there is also an additional silent substitution at nucleotide 1,455 starting from the ATG start codon of the cDNA sequence. The amino acid sequence of the *pik1* cDNA coding region is identical to that of the predicted polypeptide, based on the published genomic sequence.

The pREP vectors contain the *S. pombe nmt1* promoter (*nmt* stands for *no message on thiamine*) which is repressed when cells are grown in the presence of 5 µg/mL thiamine. When the cells are washed free of thiamine, the effects of ectopic expression can be studied. Three pREP vectors were used in this study: pREP1, pREP41 and pREP81. The pREP1 vector contains the wild-type *nmt1* promoter, while the pREP41 and pREP81 vectors have attenuated versions of the *nmt1* promoter. Both the pREP41 and pREP81 vectors provide reduced levels of expression, achieved through mutations in the TATA box of the *S. pombe nmt1* promoter (Basi *et al.*, 1993). The activity of the *nmt1* promoter, and its attenuated counterparts, have been

characterized using β -galactosidase activity assays (Forsburg, 1993). Even in the presence of thiamine, all the promoters supported some transcriptional activity. If pREP81 under repressed conditions produces 1 unit of gene activity, and assuming that the results for *lacZ* activity are generally applicable, the vector series offers the range of gene expression levels shown in Table 4.1.

Table 4.1.

Gene expression levels of 3 pREP vectors

Vector	Thiamine (+/-)	Units
pREP81	+	1
pREP81	-	6
pREP41	+	6
pREP41	-	144
pREP1	+	720
pREP1	-	44,000

The ectopic expression studies of the *pik1* wild-type and mutant alleles were performed in *S. pombe* cells that retained the intact chromosomal *pik1* gene. The *S. pombe* cells were grown in the absence of thiamine and analyzed for the accumulation of the wild-type and mutant Pik1p protein by western blot. Lipid kinase activity assays were performed on cell homogenates. Cdc4p-binding activity was assessed in yeast two-hybrid assays and by ELISA using purified Cdc4p and homogenates of cells in which the *pik1* alleles were ectopically expressed.

4.3. Generation of mutant alleles

Sequence similarity was used to identify residues that might be essential for lipid kinase activity. There is a conserved aspartic acid (D) residue in the DRH motif that is present in the p110 PtdIns 3-kinase, Vps34p (PtdIns 3-kinase) and Tor2p proteins (a dual specificity protein and lipid kinase, target of rapamycin) (Schu *et al.*, 1993; Dhand *et al.*, 1994a and b; Schmidt *et al.*, 1996). Mutation of the conserved D residue in the DRH motif of the p110 PtdIns 3-kinase, Vps34p and Tor2p proteins was shown to abolish lipid kinase activity (Schu *et al.*, 1993; Dhand *et al.*, 1994a and b; Schmidt *et al.*, 1996). Indeed, this region is involved in ATP binding and phosphotransfer. The DRH motif also exists in the type III β PtdIns 4-kinases described in Section 1.2.3. The D709 residue of Pik1p was identified as a homologous and conserved residue in this motif and was mutated to alanine (D709A) through site-directed mutagenesis (Figure 4.1). In order to determine if this allele was impaired for lipid kinase activity, assays were performed on homogenates of cells in which this allele (*pik1*^{D709A}) had been ectopically expressed.

Another goal of this study was to identify candidate residues for mutation to produce an allele impaired for binding to Cdc4p. Cdc4p interacts with the IQ domains of type II myosins in *S. pombe* (Naqvi *et al.*, 1999). A critical residue in the IQ-motif of myosins was identified as being essential for the Cdc4p interaction with Myo2p (Naqvi *et al.*, 1999). A potentially equivalent residue in a putative IQ-motif near the C-terminal end of Pik1p was identified by inspection of a sequence alignment between the myosin IQ-motif and this region of Pik1p. Within this motif (IQxxxRGxxxR), the terminal conserved arginine (R), corresponding to residue 838, was targeted for site-

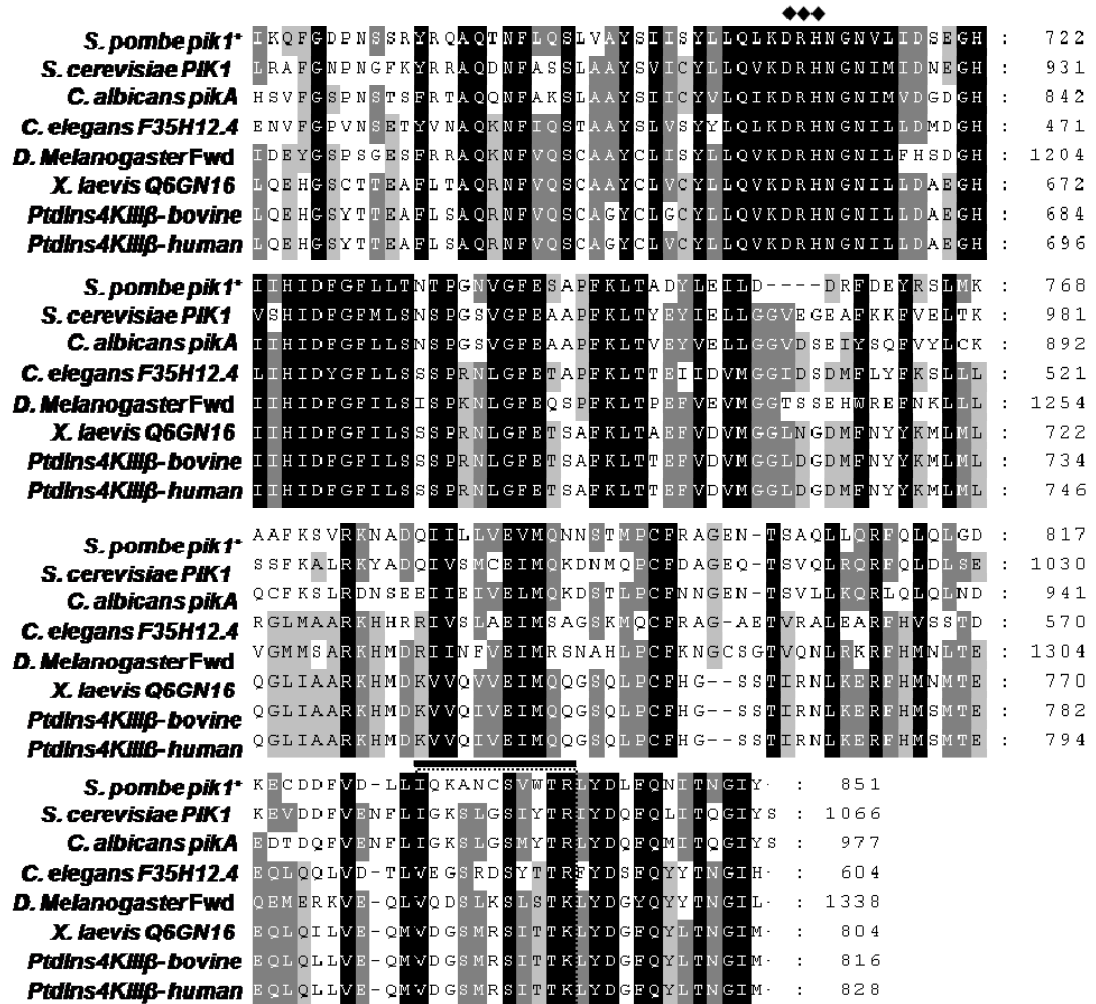


Figure 4.1: The C-terminal region of *S. pombe* Pik1p is conserved across eukaryotic PtdIns 4-kinases.

A multiple sequence alignment of the C-terminal regions of *S. pombe* Pik1p (CAA93903) orthologs in *S. cerevisiae* (CAA53658), *C. albicans* (CAA09718), *C. elegans* (NP_508177), *D. melanogaster* (NP_728519), *X. laevis* (Q6GN16), bovine (002810) and human (BAA21661). Sequences were acquired from Genebank (MM/YY, 06/05). The lipid kinase DRH motif is marked by black diamonds and the extreme C-terminal IQ-motif (IQxxxRGxxxR) is marked by a black band and dotted box.

directed mutagenesis (Figure 4.1). Thus, a second allele was created for study of *pik1* function, *pik1*^{R838A}, with the hope that it would be impaired for Cdc4p-binding activity. To analyze the Cdc4p-binding activity of the protein products produced by the *pik1*^{R838A} and *pik1*^{D709A} alleles, two approaches were utilized: (A) yeast two-hybrid assays with the C-terminal 345 amino acids of the *pik1*^{wt}, *pik1*^{D709A} and *pik1*^{R838A} alleles; and (B) ELISAs utilizing purified Cdc4p protein and homogenates of cells after the ectopic expression of the *pik1*^{wt}, *pik1*^{R838A}, *pik1*^{D709A} and *pik1*^{D709A, R838A} alleles.

4.3.1. Phylogenetic analysis of PtdIns 4-kinases and other lipid kinases

Pik1p is a putative PtdIns 4-kinase based on sequence comparisons of its primary structure with those of known PtdIns 4-kinases. In order to illustrate this fact, multiple sequence alignments were performed of sequences of known PtdIns kinases. The sequences of the kinase domains of PtdIns 4-kinases and other known lipid kinases were obtained from Pfam (<http://pfam.sanger.ac.uk/>). The sequences were analyzed for similarity and a phylogenetic tree was constructed for clustering analysis. At first glance, there are 3 major clusters: one cluster is composed of the Lsb6p-like proteins, which are type II PtdIns 4-kinases; another cluster is composed of the Fab1p-like proteins, which are PtdIns 5-kinases; and a final large cluster, which is composed of several sub-clusters, is composed of the PtdIns 3-kinases and the type III PtdIns 4-kinases (Figure 4.2). Within the large cluster, there are 4 sub-clusters: 2 clusters are composed of the PtdIns 3-kinases (A. Tor1p and Tor2p-like proteins; and B. Vps34p-like proteins) of which both groups are biochemically and biologically distinct. The other 2 clusters are composed of the type III α PtdIns 4-kinases and the type III β PtdIns

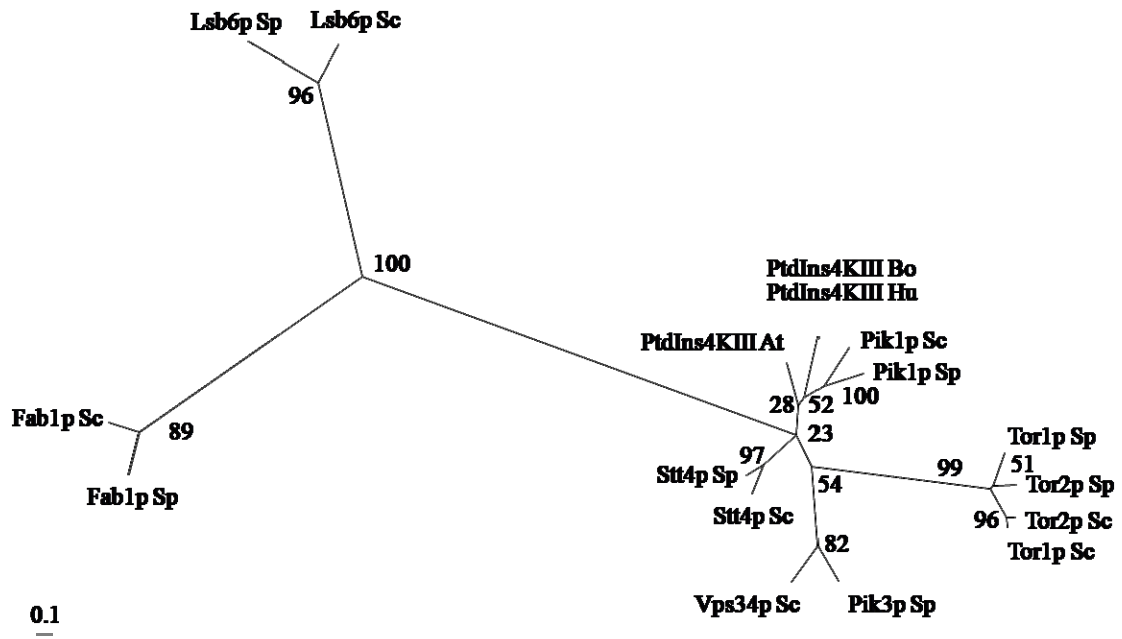


Figure 4.2: **Phylogenetic tree of PtdIns kinases.**

The kinase domains of PtdIns 3-kinases, PtdIns 4-kinases and PtdIns 5-kinases were aligned using ClustalW and a phylogenetic tree constructed using the PHYLIP software package. *S. pombe* *Pik1p* clusters with the type III β PtdIns 4-kinases and not with the PtdIns 3-kinases, PtdIns 5-kinases and type II PtdIns 4-kinases. This provides compelling evidence that *Pik1p* is type III β PtdIns 4-kinase.

4-kinases which are biochemically distinct as discussed in the literature review of this thesis. As expected, Pik1p grouped with the human, bovine, *A. thaliana* and *S. cerevisiae* type III β PtdIns 4-kinases (Figure 4.2). Based on the clusters in the phylogenetic tree, the proteins which included PtdIns 3-kinases, PtdIns 4-kinases and PtdIns 5-kinases, clustered based on the activities and biochemical properties of each of the protein groups. Overall, these results provide compelling evidence that Pik1p is a PtdIns4-kinase III β homolog based on sequence comparisons with known type III β PtdIns 4-kinases and other PtdIns kinases.

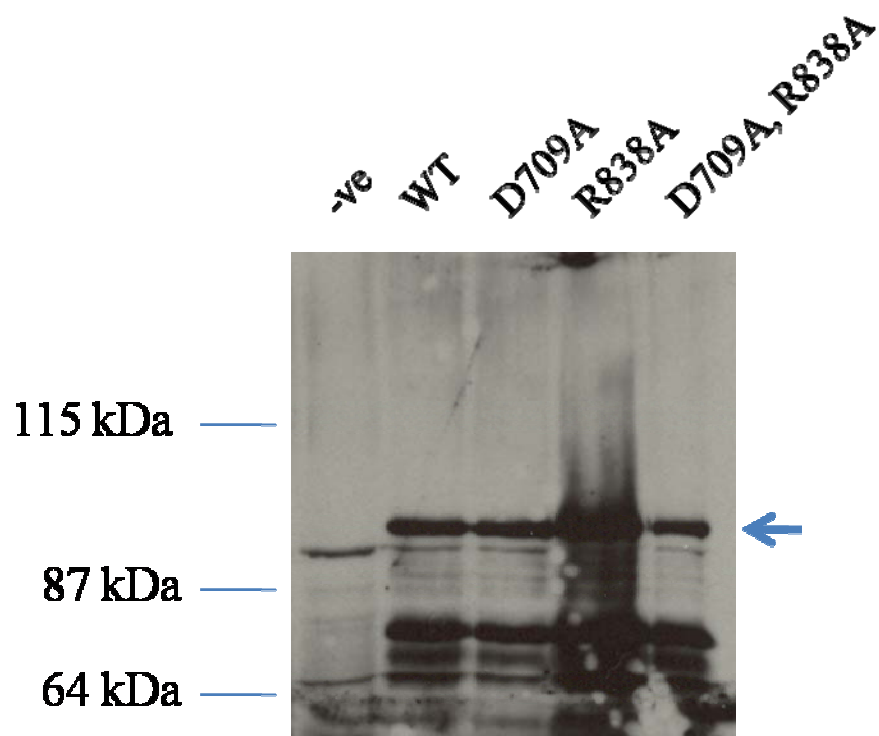
4.4. The Pik1p wild-type and mutant proteins accumulate in *S. pombe* cells in response to thiamine removal

The wild-type and mutant proteins were assayed by western blot to determine if they accumulated in *S. pombe* cells. Cells carrying the empty pREP1 vector and the pREP1 vector with the *pik1^{wt}* cDNA were grown for 24 hours at 30°C. Homogenates of cells were then made as described in the Materials and Methods (Section 3.2.10). In immunoblots of homogenates of cells carrying the empty vector in derepressed conditions, the polyclonal antibody directed against the C-terminal half of Pik1p recognized a polypeptide of 93 kDa (Figure 4.3A). In immunoblots of homogenates of cells carrying the pREP1-*pik1^{wt}* cDNA in derepressed conditions, the antibody recognized the ~93 kDa band and a strong protein band of approximately 97 kDa (Figure 4.3A). 97 kDa is the predicted molecular weight of the Pik1p protein, and the strong band observed upon the ectopic expression of the *pik1^{wt}* sequence likely corresponded to Pik1p since the presence of this band was dependent on thiamine

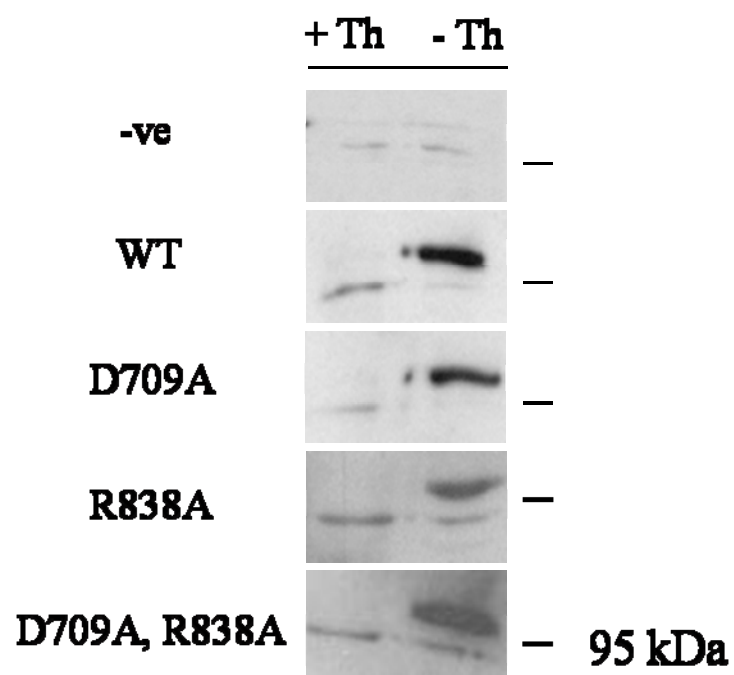
Figure 4.3: Pik1p wild-type and mutant alleles accumulate in *S. pombe* cells.

(A) The cells were transformed with pREP1 plasmid with: no insert (-ve), wild-type *pik1* (WT), *pik1*^{D709A} (D709A), *pik1*^{R838A} (R838A) and *pik1*^{D709A, R838A} (D709A, R838A). The cells were cultured for 24 hours at 30°C in the absence of thiamine. Aliquots of cell homogenates (5 µg of total protein) were subjected to denaturing polyacrylamide gel electrophoresis and immunoblot analysis with a rabbit anti-Pik1p primary antibody and a secondary HRP-conjugated antibody. The bars on the left indicate migration of the benchmark (Invitrogen) standard. Results are representative of 3 independent experiments. The arrow points to the ectopically expressed *pik1* protein product of 97 kDa. (B) The cells were transformed with pREP1 plasmid with: no insert (-ve), wild-type *pik1* (WT), *pik1*^{D709A} (D709A), *pik1*^{R838A} (R838A) and *pik1*^{D709A, R838A} (D709A, R838A). The cells were cultured for 24 hours at 30°C in the presence (+) or absence (-) of thiamine (Th). Aliquots of cell homogenates (5 µg of protein) were subjected to denaturing polyacrylamide gel electrophoresis as described above. The bars on the right indicate migration of a 95 kDa standard. Results are representative of 3 independent experiments.

A.



B.



(Figure 4.3B). Furthermore, the Pik1p cDNA encoded the same amino acid sequence as the predicted endogenous protein. The lower molecular weight band observed in homogenates of cells carrying the empty pREP1 vector likely represented a cross-reactive polypeptide. However, I cannot exclude the possibility that it may have represented the endogenous protein since it was similar in size to Pik1p. For example, the 93 kDa band may have represented a post-translationally modified form of Pik1p that migrated differently from the primary translation product. However, when *pik1^{wt}* was ectopically expressed, the lower molecular weight band displayed only a moderately lesser intensity suggesting that it was not the endogenous protein. This may have also meant that the ectopically expressed protein did not get properly processed into the lower molecular weight form. In repressed conditions, the antibody recognized the ~93 kDa protein only (Figure 4.3B). If the lower molecular weight band was indeed non-specific, this result indicated that the endogenous level of accumulation of Pik1p is just at or below the level of detection of the protein in this assay. Furthermore, these results indicated that protein accumulation was thiamine responsive, as expected for gene expression under the control of the *nmt1* promoter. In cell homogenates after the ectopic expression of *pik1^{wt}*, additional lower molecular weight bands in the range of 60 to 70 kDa were observed (Figure 4.3A). Most of these polypeptides likely represented break-down products of ectopically expressed *pik1^{wt}* since they were not present in cell homogenates carrying the vector alone. Despite the addition of protease inhibitors, apparently degradation still occurred in the homogenates. However, there was a 67 kDa band present in the cell homogenates carrying the vector alone. See below for a description.

Immunoblots of homogenates of cells carrying the pREP1-*pikI*^{D709A} vector indicated that the Pik1p^{D709A} protein also accumulated in cells under derepressed conditions (Figure 4.3A). This is in contrast to homogenates of cells carrying the *pikI*^{D709A} allele grown in repressed conditions or cells carrying the vector alone where no protein band of 97 kDa was observed (Figure 4.3A and B). Thus, both the Pik1p wild-type and Pik1p^{D709A} proteins accumulated over 24 hours at 30°C. The *pikI*^{R838A} and *pikI*^{D709A,R838A} proteins also accumulated in *S. pombe* cells after 24 hours of growth at 30°C in derepressed conditions. Apparent levels of Pik1p^{R838A} protein in cell homogenates were reproducibly increased compared to the other *pikI* alleles.

As expected, there was no observed accumulation of these proteins in repressed conditions. The 93 kDa band was observed in all homogenates of cells prepared (Figure 4.3A and B). Furthermore, lower molecular weight polypeptides within the range of 60 to 70 kDa were also recognized by the antibody in homogenates of cells after which the *pikI*^{D709A}, *pikI*^{R838A} and *pikI*^{D709A,R838A} alleles were ectopically expressed (Figure 4.3A). As explained above for the *pikI*^{wt} protein, these lower molecular weight bands likely represented the break-down products of the mutant Pik1p proteins as they were absent in cell homogenates carrying the vector alone. However, there was a band of approximately 67 kDa present in homogenates of cells carrying the vector alone, and when the *pikI* alleles were ectopically expressed, this band increased in intensity. This 67 kDa band may represent an alternative isoform of Pik1p or may represent a degradative product of the endogenous 93 kDa Pik1p protein. In order to determine if the 93 kDa band was the endogenous protein, western blot analysis with homogenates of cells deleted for the *pikI* locus and rescued with a *pikI*-2xeGFP

episome, could be performed. If the 93 kDa band was not observed in homogenates of cells deleted for *pik1*, then it might be concluded that the 93 kDa band was derived from the endogenous protein.

It remained to be established if the Pik1p protein produced by ectopic expression of *pik1^{wt}* is enzymatically active and whether the D709A mutation effectively impaired its lipid kinase activity. Furthermore, the effects of the R838A mutation on *pik1* lipid kinase activity were unknown. Thus, a lipid kinase assay was developed to assess the lipid kinase activity of the *pik1^{wt}*, *pik1^{D709A}*, *pik1^{R838A}* and *pik1^{D709A,R838A}* alleles.

4.5. The lipid kinase activity of the *pik1* alleles

4.5.1. Pik1p is catalytically active

A lipid kinase assay (see section 3.2.14) was required to determine if the wild-type *pik1* allele encodes a lipid kinase and if the *pik1^{D709A}* mutation affected its activity. Also, the effects of the R838A mutation on *pik1* lipid kinase activity needed to be assessed. I attempted to adapt a published assay (Mahon *et al.*, 2005) that used exogenously added PtdIns/PS (phosphatidylserine) lipid micelles and ³²P-γ-ATP as substrates. The formation of the radiolabeled lipid product was also observed to be independent of the exogenously added substrate. Since the exogenously added PtdIns was derived from mammalian cells, this may have reflected an inability of the Pik1p protein to recognize vertebrate derived PtdIns. Vertebrate PtdIns is polyunsaturated, whereas fungal derived PtdIns is not (Veen and Lang, 2004). I therefore modified the assay. The modified assay used in this study measured the incorporation of ³²P into endogenous PtdIns substrate.

S. pombe cells transformed with the pREP1 vector carrying the *pik1* wild-type and mutant alleles were cultured for 24 hours at 30°C in the presence and absence of thiamine. Cell homogenates were made as described in the Materials and Methods. After estimating the total protein content, aliquots containing amounts of protein over the range of 0.05 – 0.8 µg were prepared. ³²P-γ-ATP was then added to each aliquot and the samples incubated for 15 minutes at room temperature. Following the incubation with ³²P-γ-ATP, the acidic and polar phospholipids were extracted, and the recovered lipids spotted onto a thin layer chromatography (TLC) plate. After separation of the lipids by TLC, the plates were then either exposed to film or the thiamine-responsive radiolabeled lipid product was recovered from the TLC plate by scraping and ³²P radioactivity was determined by liquid scintillation counting.

As seen in Figure 4.4, in the absence of thiamine, there was a marked increase in ³²P activity associated with the PtdIns product that co-migrated with a PtdIns4P lipid-standard, and not with products that co-migrated with the PtdIns, PtdIns(4,5)P₂ or PtdIns(3,4,5)P₃ lipid standards. The chromatography system used did not resolve PtdIns3P from PtdIns4P, and thus it can be concluded that the major radiolabeled lipid product was a monophosphorylated version of PtdIns.

To better define the product of *pik1*^{wt} lipid kinase activity, heterologous expression studies were undertaken in *E. coli* BL21(DE3) cells. However, expression of *pik1*^{wt} in *E. coli* produced an insoluble protein product. Lipid kinase assays were also performed after immunoprecipitating the Pik1p protein from *E. coli* and *S. pombe* cell homogenates using a polyclonal antibody directed against Pik1p (Desautels *et al.*, 2001). Attempts at this approach were not successful. The antibody used was directed

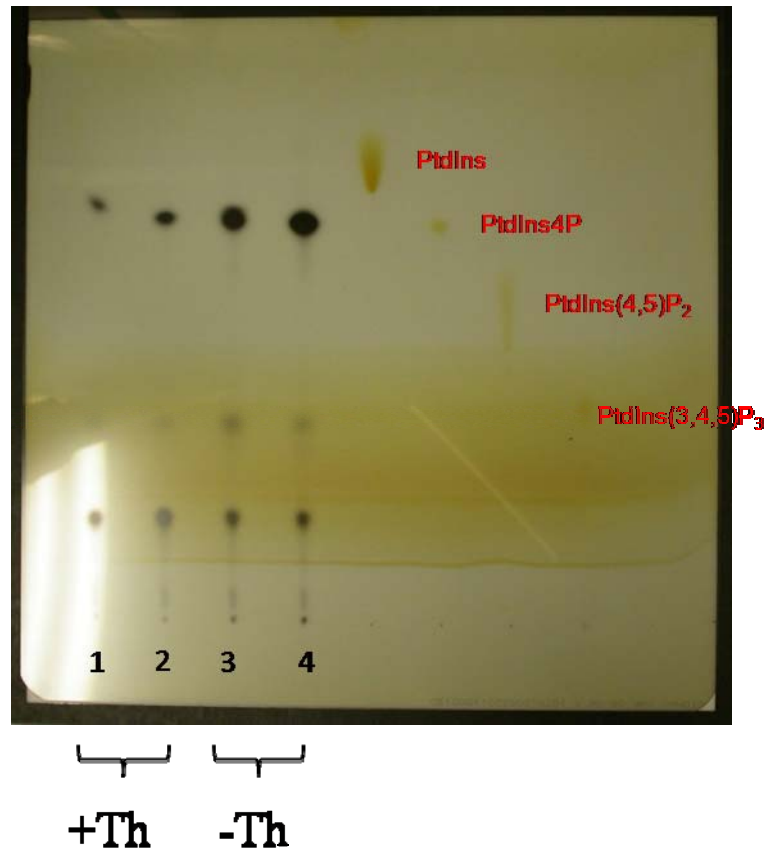


Figure 4.4: Ectopically expressed *pik1^{wt}* is catalytically active.

Autoradiograph of thin layer chromatogram after resolution of radioactive products from *in vitro* lipid kinase assays. *S. pombe* cells were transformed with the pREP1 expression vector carrying the full-length wild-type *pik1* cDNA coding sequence under the control of the *nmt1* promoter. These cells were cultured for 24 hours at 30°C in the presence (lanes 1 and 2) and absence (lanes 3 and 4) of thiamine. Aliquots of cell homogenates that contained 0.8 µg of total protein were pulse labelled with ³²P-γ-ATP. Acidic phospholipids were extracted and subjected to thin layer chromatography. Known reference lipid standards (50 µg of each) were separated on the TLC plate along side the lipid product from the activity assay. An increase in ³²P-γ-ATP incorporation into the lipid spot is observed in the absence of thiamine. This radiolabeled lipid product co-migrated with the PtdIns4P lipid standard.

against the C-terminal half of Pik1p, which contained the lipid kinase domain. Thus, it is possible that the antibody interfered with the assay. Also, the Pik1p antibody was raised against completely denatured Pik1p protein, thus, it is unknown as to the efficiency of recognition by the antibody to the protein in the crude homogenate. Further studies to determine the efficiency of pulling down the Pik1p protein with this antibody should be performed.

The incorporation of ^{32}P into monophosphorylated PtdIns in homogenates of cells carrying the vector alone increased in a linear fashion over the range of 0.05-0.8 μg of total protein and was approximately the same in both repressed and derepressed conditions (Figure 4.5). This labeled monophosphorylated PtdIns may have been produced by the Pik1p protein, but the activities of the Stt4p-like PtdIns 4-kinase and the Lsb6p-like PtdIns 4-kinase may have also contributed to it. Since the lipid kinase activity assays were performed using crude cell homogenates, it cannot be ruled out that the labeled PtdInsP lipid product could be the net effect of all the other PtdIns 3-kinases, PtdIns 4-kinases and phosphatases in the homogenate.

The incorporation of ^{32}P into monophosphorylated PtdIns in homogenates of cells in which the *pik1* wild-type sequence had been heterologously expressed was then assessed in both repressed and derepressed conditions (Figure 4.5). In repressed conditions, the incorporation of ^{32}P into monophosphorylated PtdIns increased in a linear fashion over the range of 0.05-0.8 μg of protein (Figure 4.5). This activity was slightly higher than that of homogenates of cells carrying the vector alone grown in repressed and derepressed conditions (~1.3-fold higher in activity per μg of input protein) (Figure 4.5). The difference in activity of the homogenates of cells carrying the

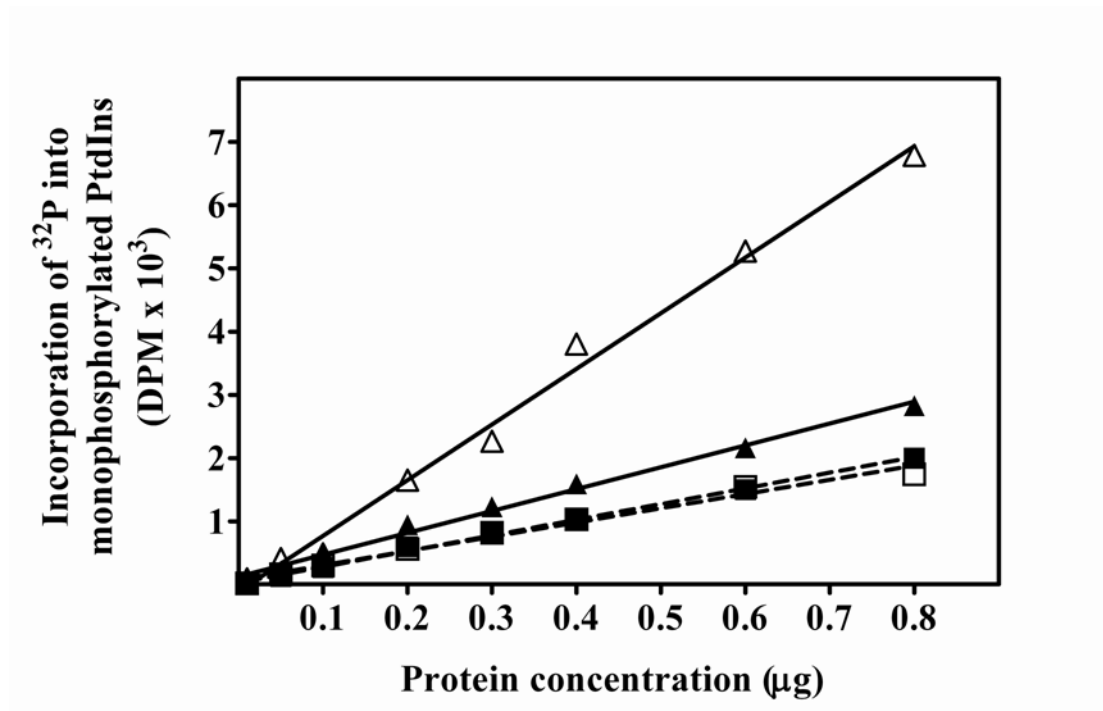


Figure 4.5: **Ectopically expressed *pik1* in *S. pombe* is catalytically active.**

S. pombe homogenates of cells transformed with the pREP1 empty vector and pREP1 vector containing the *pik1* cDNA were pulse labelled with ^{32}P - γ -ATP and the acidic phospholipid products separated by TLC. The major radiolabeled lipid spot was scraped and radioactivity measured by liquid scintillation counting. Homogenates were from cells carrying the wild-type *pik1* sequence (solid lines with open or closed triangles) or from cells carrying the empty vector (dotted lines with open or closed squares). Culture medium was supplemented with thiamine (repressed expression, solid symbols) or not (derepressed expression, open symbols). Results are representative of 3 independent experiments.

vector alone and the homogenates of cells carrying the *pik1^{wt}* allele and grown in repressed conditions, likely represents the lipid kinase activity from the leaky expression of *pik1^{wt}* from the *nmt1* promoter. In derepressed conditions, the incorporation of ³²P into monophosphorylated PtdIns of homogenates of cells carrying ectopically expressed *pik1^{wt}* again increased in a linear fashion over the range of 0.05-0.8 µg of protein (Figure 4.5). The activity per µg of input protein was ~3.8-fold greater than that of cells carrying the vector alone, and ~2.8-fold higher than that of cells carrying the *pik1* wild-type sequence grown in repressed conditions (Figure 4.5). These results are representative of the two experiments of this type that were performed. Thus, ectopically expressed Pik1p accumulates in *S. pombe* cells and is catalytically active.

4.5.2. The Pik1p^{D709} residue is required for Pik1p lipid kinase activity

In order to determine whether the *pik1^{D709A}* mutation impaired *pik1^{wt}* lipid kinase activity, homogenates of cells carrying the *pik1^{D709A}* allele were assayed for the incorporation of ³²P into monophosphorylated PtdIns. Cells carrying the pREP1-*pik1^{D709A}* vector were cultured in repressed and derepressed conditions for 24 hours at 30°C. After a cell homogenate was made as described in the Materials and Methods (Section 3.2.10), lipid kinase activity was observed by either generating an autoradiograph of the TLC plate, or the radiolabeled lipid product scraped, measured by liquid scintillation counting and a graph of the incorporation of ³²P into monophosphorylated PtdIns plotted. An increase in the incorporation of ³²P into monophosphorylated PtdIns upon the ectopic expression of *pik1^{wt}* was first observed on

an autoradiograph. Cell homogenates containing 0.05 µg of input protein that were pulse labeled with ^{32}P - γ -ATP produced more radiolabeled PtdInsP product in derepressed conditions than in repressed conditions (Figure 4.6A). In the case of the *pik1^{D709A}* mutant, 0.05 µg of cell homogenates pulse labeled with ^{32}P - γ -ATP produced less radiolabeled lipid PtdInsP product in derepressed conditions than in repressed conditions or compared to cell homogenates carrying ectopically expressed *pik1^{wt}* (Figure 4.6A).

The incorporation of ^{32}P into monophosphorylated PtdIns of homogenates of cells carrying the *pik1^{D709A}* allele in repressed conditions increased in a linear fashion over the range of 0.05-0.8 µg of protein to levels comparable to homogenates of cells carrying the vector alone (Figure 4.6B). However, in derepressed conditions, the activity per µg of input protein was approximately 1.6-fold lower than that of the same cells grown in repressed conditions (Figure 4.6B). All together, western blot analysis indicates that the ectopic expression of the *pik1^{D709A}* sequence causes the protein product to accumulate in *S. pombe* cells in the absence of thiamine, but this protein product does not appear to have lipid kinase activity.

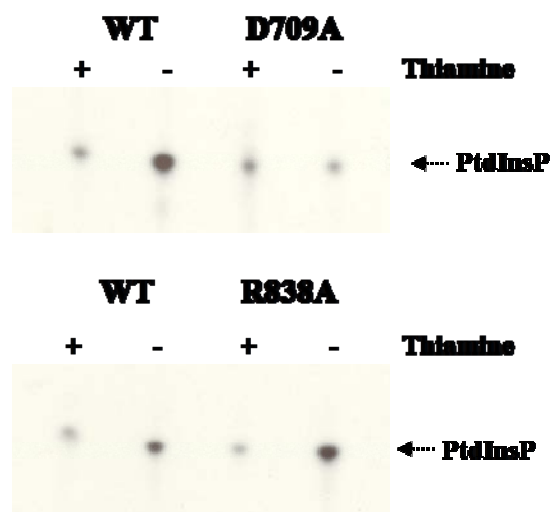
4.5.3. The Pik1p R838 residue is not required for Pik1p lipid kinase activity

Pik1p^{R838A} accumulates upon ectopic expression. It was then of interest to evaluate if the Pik1p^{R838A} protein had altered lipid kinase activity. Cells transformed with the pREP1 plasmid carrying the *pik1^{R838A}* sequence were cultured in the presence or absence of thiamine at 30°C for 24 hours. Examination of an autoradiogram of the TLC plate indicated that 0.05 µg of cell homogenate carrying the ectopically expressed

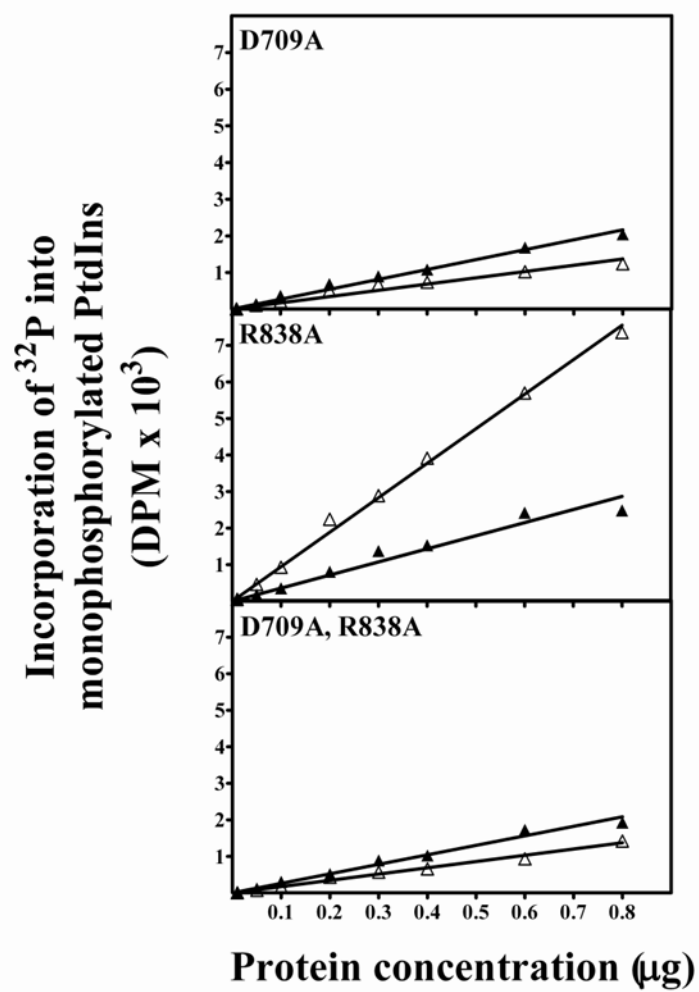
Figure 4.6: The *pik1* D709 residue is required for *pik1* lipid kinase activity, but the R838 residue is not.

(A) Autoradiograph of thin layer chromatogram after resolution of radioactive products from *in vitro* lipid kinase assays. *S. pombe* cells were transformed to leucine prototrophy with pREP1 expression vectors carrying the *pik1* full-length wild-type and mutant *pik1*^{D709A} and *pik1*^{R838A} cDNA coding sequences under the control of the *nmt1* promoter. Cells were cultured for 24 hours at 30°C in the presence (+) and absence (-) of thiamine. Aliquots of cell homogenates (0.05 µg of total protein) were pulse labelled with ³²P-γ-ATP. Acidic phospholipids were extracted and subjected to thin layer chromatography. The spot marked PtdInsP was identified as ³²P-labeled monophosphorylated PtdIns, by reference to lipid standards. Results shown are representative of 3 independent experiments. (B) Analysis of the production of ³²P-labeled monophosphorylated PtdIns by the ectopic expression of *pik1*^{D709A}, *pik1*^{R838A} and *pik1*^{D709A, R838A}. Homogenates were from cells transformed with the pREP1 expression vector carrying the *pik1*^{D709A}, *pik1*^{R838A} and *pik1*^{D709A, R838A} sequences. Cells were cultured for 24 hours at 30°C. Culture medium was supplemented with thiamine (repressed expression, solid symbols) or not (derepressed conditions, open symbols). Results shown are representative of 3 independent experiments.

A.



B.



Pik1p^{R838A} protein incorporated equal, if not more, ³²P-γ-ATP into monophosphorylated PtdIns than the wild-type sequence (Figure 4.6A). This activity was also higher than that in cells carrying the same sequence, but grown in repressed conditions. In derepressed conditions, the activity per μg of input protein was ~2.3-fold that of cells cultured in the presence of thiamine and ~3.9-fold of that of cell homogenates carrying the vector alone (Figure 4.6B). This incorporation of ³²P into monophosphorylated PtdIns is comparable to that of homogenates of cells carrying the *pik1* wild-type sequence in derepressed conditions (compare Figure 4.5 with Figure 4.6B). Thus, the *pik1*^{R838A} allele accumulates in protein product and is catalytically active. However, western blot analysis indicates that this allele accumulates to a greater extent than the other *pik1* alleles in wild-type *S. pombe* cells. Thus, the increased lipid kinase activity over the wild-type allele may have been due to the increased stability of the *pik1*^{R838A} protein.

The lipid kinase activity of the *pik1*^{D709A, R838A} allele was also assessed. Under repressed conditions, the incorporation of ³²P into monophosphorylated PtdIns in homogenates of cells ectopically expressing the *pik1*^{D709A, R838A} allele increased linearly over the range of 0.05-0.8 μg of total protein and was slightly reduced compared to the *pik1*^{wt} allele under similar conditions (compare Figure 4.5 with Figure 4.6B). In derepressed conditions, the activity per μg of input protein decreased approximately 1.8-fold relative to that of cells grown in repressed conditions (Figure 4.6B). Thus, like the ectopic expression of the *pik1*^{D709A} allele, ectopic expression of the double-mutant results in decreased lipid kinase activity in cell homogenates. The lack of incorporation

of ^{32}P into monophosphorylated PtdIns upon the expression of this allele is not due to protein instability as the polypeptide was observed to accumulate in *S. pombe* cells.

In summary, the *pik1* wild-type sequence is catalytically active, while the *pik1*^{D709A} and *pik1*^{D709A,R838A} sequences are not. The *pik1*^{R838A} mutation has little to no effect on Pik1p lipid kinase activity. The observed increase in lipid kinase activity of the *pik1*^{R838A} allele may have been due to increased stability of the *pik1*^{R838A} protein in cell homogenates.

4.6. The Cdc4p binding activity of the *pik1* alleles

4.6.1. The Cdc4p interaction with the C-terminal 345 amino acids of Pik1p^{R838A} is not observed in a yeast two-hybrid assay

The *pik1*^{R838} residue was identified as a conserved residue in a putative IQ-motif present in the C-terminal region of Pik1p. This residue was mutated to alanine in hopes of generating a *pik1* allele that could not bind to Cdc4p. Also, the *pik1*^{D709A} mutation, which abolished lipid kinase activity, has unknown Cdc4p-binding activity. To determine whether the *pik1*^{R838A} and *pik1*^{D709A} alleles were able to bind Cdc4p, these mutations were introduced into the *pik1* cDNA in the yeast two-hybrid pBI771 vector. In this assay, the region of *pik1* encoding residues 507-851 (*C-pik1*) was fused to the Gal4p transactivating (TA) domain and used to transform YPB2 *S. cerevisiae* cells to tryptophan prototrophy. The same YPB2 cells were then transformed to leucine prototrophy with a vector (pBI880) that fused the Gal4p DNA binding domain (DBD) to the full-length *cdc4* coding region. Reconstitution of the Gal4p transcription factor with a Pik1p-Cdc4p interaction results in the expression of two genes: *HIS3* and *lacZ*.

This allows the cells to grow on plates lacking histidine, in the presence of 5 mM 3-AT, and the colonies to turn blue in the presence of X-gal substrate, respectively.

Before assessing the Cdc4p-binding activity of the *pik1*^{R838A} and *pik1*^{D709A} alleles, a negative control was performed to ensure that the Gal4p DBD alone or the *C-pik1*-Gal4p-TA construct were not self-activating; that is, that growth on plates lacking histidine was not observed. To confirm that the colonies contained the presence of both plasmids, colony formation assays were performed on SD media lacking leucine and tryptophan, which selects for both plasmids. The colony formation of YPB2 cells carrying both the empty 'bait' vector (pBI880, no *cdc4* insert) and the 'prey' construct (pBI771 with *C-pik1*) was observed on SD –Leu –Trp plates (data not shown). However, these cells did not form colonies on SD –Leu –Trp –His +3AT plates as is expected since a productive interaction cannot occur in these cells (Figure 4.7A). This also shows that the Gal4p DBD alone or the Gal4p TA-*C-pik1* fusion proteins are unable to cause *HIS3* or *lacZ* activation. Additional controls were also performed. Cells carrying the empty 'bait' vector were co-transformed with the empty 'prey' vector and 'prey' vectors carrying the *pik1* wild-type and mutant alleles. Again, these cells formed colonies on plates that selected for the presence of both plasmids (SD –Leu –Trp), but did not grow on plates that selected for the protein-protein interaction (SD –Leu –Trp –His +3-AT) (data not shown).

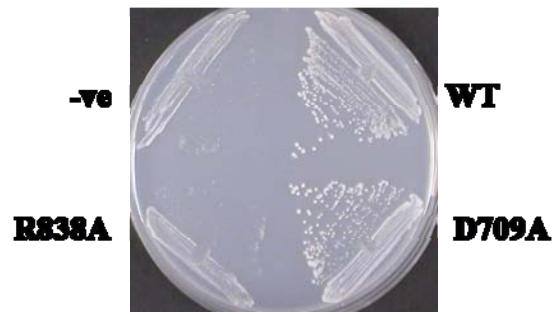
Cdc4p interacts with the C-terminal region of Pik1p as previously established (Desautels *et al.*, 2001). Thus, the wild-type *C-pik1* sequence was used as a positive control. The colonies carrying both the 'bait' (pBI880 with Cdc4p N-terminally fused to the Gal4p DBD) and the 'prey' (pBI771 with *C-pik1* N-terminally fused to the Gal4p

Figure 4.7: The *pik1* R838 residue is required for the interaction with Cdc4p in a yeast two-hybrid assay.

(A) Yeast two-hybrid protein-protein colony formation assay. *S. cerevisiae* cells transformed with a two-hybrid bait vector (pBI880) carrying the *S. pombe cdc4* sequence fused to the *GAL4* DNA binding domain were co-transformed with two-hybrid prey vectors (pBI771) carrying the C-terminal 345 amino acids of the *S. pombe pik1* coding sequence fused to the *GAL4* transcription activation domain. Cells from single colonies of co-transformants were plated on medium that selected for each plasmid and for the protein-protein interaction (histidine prototrophy and resistance to 3-AT (5 mM from 2 M stock)). Co-transformants carried: -ve, the empty prey vector; WT, wild-type *pik1* sequence; R838A or D709A *pik1* sequences. Results shown are representative of 3 independent experiments. (B) X-gal colony filter assays.

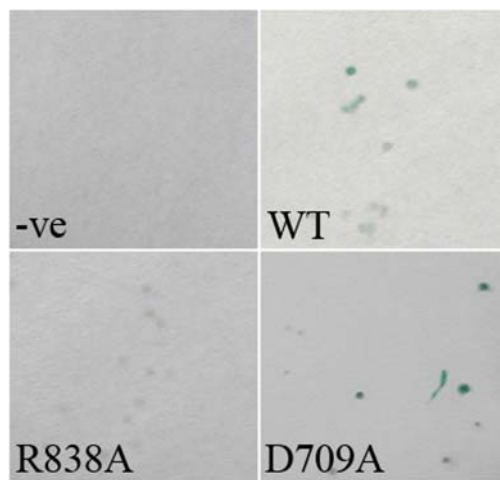
Nitrocellulose filters were overlayed onto the *S. cerevisiae* colonies grown on SD –Leu –Trp plates, submerged in liquid nitrogen and incubated in X-gal solution to monitor β -galactosidase activity. Cells carrying the *pik1* wild-type and D709A alleles fused to the *GAL4*-TA domain in the pBI771 vector and *cdc4* sequence fused to the *GAL4*-BD in the pBI880 vector turned blue and were thus positive for the Pik1p-Cdc4p interaction.

A.



B.

pBI771 *pik1* allele



TA domain) vectors grew equally well on SD plates lacking leucine and tryptophan which selected for the presence of both plasmids (data not shown). YPB2 cells carrying both the Cdc4p sequence and the C-terminal region of Pik1p formed colonies on SD – Leu –Trp –His +3AT plates, and turned blue in the presence of X-gal, indicating a positive protein-protein interaction between Pik1p and Cdc4p (Figure 4.7A and B).

Next, the Cdc4p-binding activity of the *pik1*^{R838A} mutant was assessed. Cells carrying both the ‘bait’ (pBI880) Cdc4p-Gal4p DBD fusion vector and ‘prey’ (pBI771) C-Pik1p^{R838A} Gal4p TA fusion vector formed colonies on SD media lacking leucine and tryptophan verifying that the colonies carried both plasmids (data not shown). These cells however, did not form colonies on plates which selected for the protein-protein interaction (SD media lacking leucine, tryptophan, histidine and supplemented with 3-AT) (Figure 4.7A). Thus, the Pik1p^{R838A} mutation abolished the Pik1p interaction with Cdc4p in these assay conditions. The cells that grew on SD –Leu –Trp plates were tested for β-galactosidase activity and none of the colonies turned blue suggesting that *lacZ* activation or a productive protein-protein interaction was not occurring in these cells (Figure 4.7B).

The Cdc4p-binding activity of the *pik1*^{D709A} allele is unknown. Cells carrying both the Cdc4p pBI880 vector and Pik1p^{D709A} pBI771 vector were able to form colonies on plates lacking leucine and tryptophan indicating that the cells carried both plasmids (data not shown). When the same cells were assayed for colony formation on plates that selected for the protein-protein interaction (SD media lacking leucine, tryptophan, histidine and supplemented with 3-AT), colony formation was observed (Figure 4.7A). Furthermore, the cells grown on SD –Leu –Trp plates were assayed for β-galactosidase

activity. These colonies turned blue, again indicating a positive protein-protein interaction (Figure 4.7B). Thus, Cdc4p interacted with the Pik1p^{D709A} protein. In summary, in the yeast two-hybrid assay, the R838 residue is required for the interaction with Cdc4p, while the D709 residue is not.

4.6.2. The Cdc4p interaction with the C-terminal region of Pik1p is specific for Cdc4p and not Ncs1p

A small EF-hand protein, Frq1p, was shown to interact with Pik1p in *S. cerevisiae* (Hendricks *et al.*, 1999). Frq1p is the orthologue of Ncs1p in fission yeast and Ncs1p was shown to interact with *S. cerevisiae* Pik1p (Hamasaki-Katagiri *et al.*, 2004). It was then of interest to evaluate if Ncs1p interacted with Pik1p, and if so, whether this interaction required the same structural determinants as the interaction with Cdc4p. To that aim, a *ncs1* cDNA clone was obtained by reverse transcription and cloned into pBI880 *in lieu* of *cdc4*. *S. cerevisiae* YPB2 cells carrying both the pBI771-*C-pik1* and pBI880-*ncs1* plasmids formed colonies on plates lacking both leucine and tryptophan (Figure 4.8). When the same cells were streaked onto plates selecting for the protein-protein interaction (SD –Leu –Trp –His +3AT), colonies did not form suggesting that the Gal4p-DBD-Ncs1p fusion protein did not interact with the Gal4p-TA-C-Pik1p fusion protein (Figure 4.8). As a positive control, cells co-transformed with the *cdc4* pBI880 vector and the *C-pik1* pBI771 vector were streaked on the same plates. As previously established, Cdc4p interacted with the C-terminal region of Pik1p (Figure 4.8). Thus, under similar conditions, no positive interaction between Ncs1p and the C-terminal domain of Pik1p was observed.

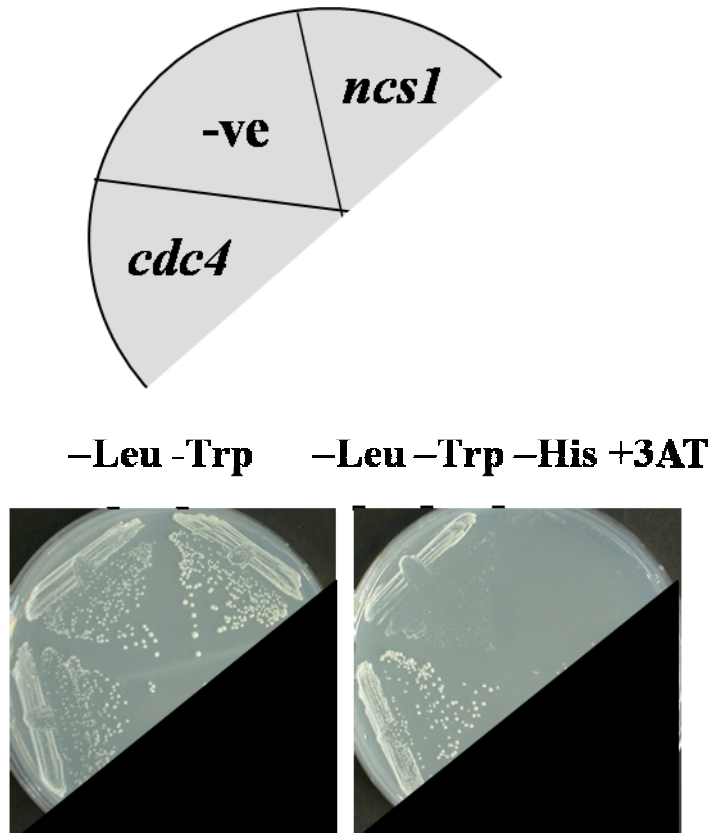


Figure 4.8: The EF-hand protein Ncs1p does not interact with the C-terminal region of Pik1p.

Yeast two-hybrid protein-protein interaction colony formation assays. *S. cerevisiae* cells transformed with a two-hybrid pBI880 vector carrying the *S. pombe cdc4* cDNA and *ncs1* cDNA sequences fused to the GAL4 DNA binding domain were co-transformed with a two-hybrid pBI771 vector carrying the *S. pombe pik1* coding sequence Pik1p (507-851) fused to the GAL4 transactivation domain. Cells from single colonies of co-transformants were plated on medium that selected for each plasmid (-Leu -Trp) and for protein-protein interaction (-Leu -Trp -His + 3AT). As a negative control, cells were co-transformed with the empty pBI880 vector (-ve). Results shown are representative of 3 independent experiments.

Western blot analysis to determine if the *pik1* fusion proteins were accumulating in the YPB2 *S. cerevisiae* cells, were unsuccessful. Attempts to detect the C-*pik1* wild-type allele were also unsuccessful suggesting that the western blot analysis was not working, even though histidine protrophy and *lacZ* expression were occurring in the YPB2 *S. cerevisiae* cells, indicating a positive protein-protein interaction. However, the *pik1*^{R838A} allele did complement the *S. cerevisiae pik1-101* temperature-sensitive mutant at the non-permissive temperature (Park, 2007). Also, western blot analysis of wild-type *S. pombe* cells showed that the *pik1*^{R838A} gene product accumulated in these cells. Thus, it is likely that the C-*pik1*^{R838A} TA fusion protein is accumulating in the YPB2 *S. cerevisiae* cells and is not interacting with Cdc4p in this assay. This may have indicated that the expression of the fusion proteins was too low to detect using our available antibodies. This will be discussed again in the discussion (Chapter 5). To confirm that the Pik1p R838A mutant abrogates the interaction with Cdc4p, ELISAs using homogenates of cells expressing the full-length Pik1p wild-type and mutant proteins were performed.

4.6.3. The Pik1p residue R838 is required for the interaction with Cdc4p in ELISAs

So far, yeast two-hybrid assays established an interaction between Cdc4p and the C-terminal region of Pik1p and Pik1p^{D709A}, but not Pik1p^{R838A}. We have yet to demonstrate an interaction with the full-length protein. Also, the removal of the Pik1p-Cdc4p interaction by the Pik1p^{R838A} mutation needed to be confirmed. To meet this objective, two assays were performed: (A) yeast two-hybrid assays with full-length

pik1^{wt}, and (B) ELISAs with purified Cdc4p and cell homogenates with expressed Pik1p protein. Yeast two-hybrid analysis was performed to determine if the full-length Pik1p protein interacted with Cdc4p. As a negative control, cells were co-transformed with the full-length *pik1* coding sequence in the pBI771 ‘prey’ vector and the empty pBI880 ‘bait’ vector. These cells formed colonies on plates selecting for the presence of both plasmids (SD – Leu – Trp), but did not form colonies on plates selecting for the protein-protein interaction (SD – Leu – Trp – His +3AT) (Figure 4.9). Cells co-transformed with the full-length *pik1* sequence and the *cdc4* sequence formed colonies on plates that selected for the presence of both plasmids (SD – Leu – Trp), but did not form colonies on plates that selected for the protein-protein interaction (SD – Leu – Trp – His +3AT) (Figure 4.9). Thus, the full-length Pik1p protein did not interact with Cdc4p in this assay. Likewise, Ncs1p was studied for its possible interaction with the full-length Pik1p protein. Ncs1p also did not interact with full-length Pik1p in this assay (Figure 4.9).

The C-terminal domain of Pik1p, but not the full-length protein, interacted with Cdc4p in the yeast two-hybrid assay. To further investigate the potential interaction between the full-length Pik1p protein and Cdc4p, as well as to analyze the effects of the *pik1^{R838}* residue on this interaction, ELISAs were performed. Cells carrying the pREP1 vector with the *pik1^{wt}* and mutant alleles were cultured in media with and without thiamine for 24 hours at 30°C. Cell homogenates were prepared as described in the Materials and Methods (Section 3.2.10). As previously established by western blot analysis, each of the products of the *pik1* alleles accumulated in the *S. pombe* cells under these conditions.

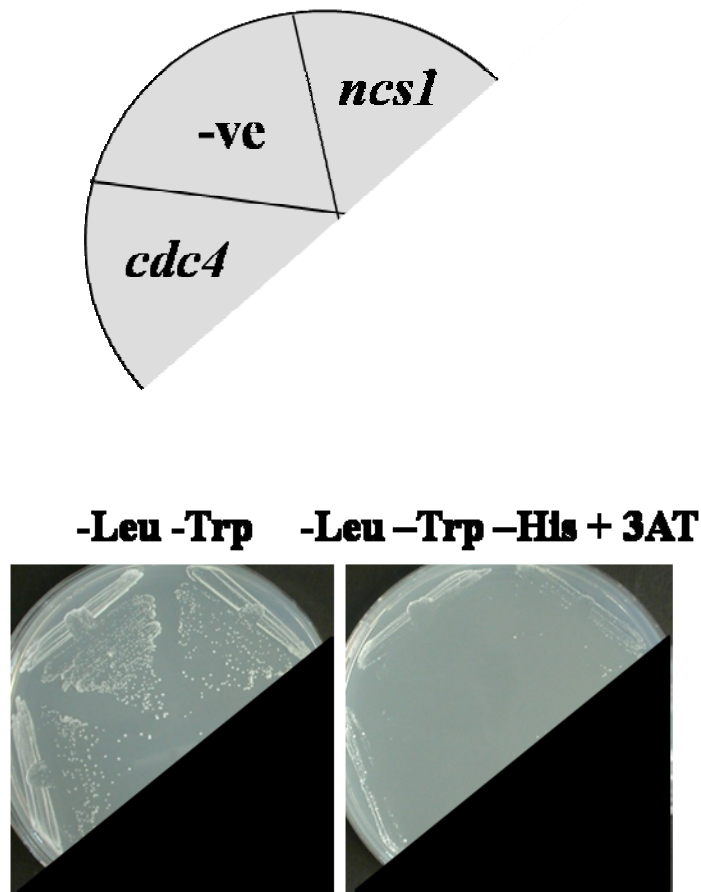


Figure 4.9: Cdc4p does not interact with the full-length version of Pik1p in a yeast two-hybrid assay.

Yeast two-hybrid protein-protein interaction colony formation assays. *S. cerevisiae* cells transformed to leucine prototrophy by a two-hybrid pBI880 vector carrying the *S. pombe cdc4* cDNA and *ncs1* cDNA sequences fused to the GAL4 DNA binding domain were co-transformed to tryptophan prototrophy by two-hybrid pBI771 vectors carrying the *S. pombe pik1* coding sequences (Pik1p 1-851) fused to the GAL4 transcription activation domain. Cells from single colonies of co-transformants were plated on medium that selected for each plasmid (-Leu -Trp) and for protein-protein interaction (-Leu -Trp -His + 3AT). As a negative control, cells were co-transformed with the empty pBI880 vector (-ve). Results shown are representative of 3 independent experiments.

For ELISA, two-fold serial dilutions of homogenates of cells carrying the expressed *pik1* alleles were made. These serially diluted cell homogenates were each then incubated in multi-well plates coated with purified Cdc4p (see Section 3.2.13.1). After washing and blocking, detection of the Pik1p-Cdc4p interaction was performed by using a primary polyclonal rabbit antiserum directed against Pik1p followed by incubation with a secondary antibody directed against rabbit IgG and conjugated to horseradish peroxidase (HRP). The Pik1p-Cdc4p interaction was then measured by colorimetric change with an ELISA plate reader upon the addition of HRP substrate. In cell homogenates carrying the expressed full-length Pik1p protein, there was a linear increase in ELISA signal with increasing input homogenate in the assay (Figure 4.10). Thus, the full-length Pik1p protein interacted with Cdc4p in a linear fashion over the range of 18-56 µg of homogenate protein (Figure 4.10). This was in contrast to the negative controls which were homogenates of cells carrying the vector alone, or homogenates of cells carrying the *pik1* wild-type sequence grown in repressed conditions (Figure 4.10). These homogenates failed to produce a significant ELISA signal under the same conditions with absorbance readings close to background (Figure 4.10). Thus, Cdc4p interacts with full-length Pik1p.

The full-length Pik1p^{D709A} protein also interacted with Cdc4p in the ELISA assay. In fact, per µg of homogenate protein, homogenates from *pik1*^{D709A} cells produced a greater ELISA signal for binding to plates coated with Cdc4p than did *pik1*^{wt} cells (Figure 4.10). This may indicate that the Pik1p^{D709A} protein accumulated to higher levels than the Pik1p wild-type protein, or that the Pik1p^{D709A} allele interacted with higher affinity to Cdc4p than the wild-type enzyme. Western blot analysis of cells

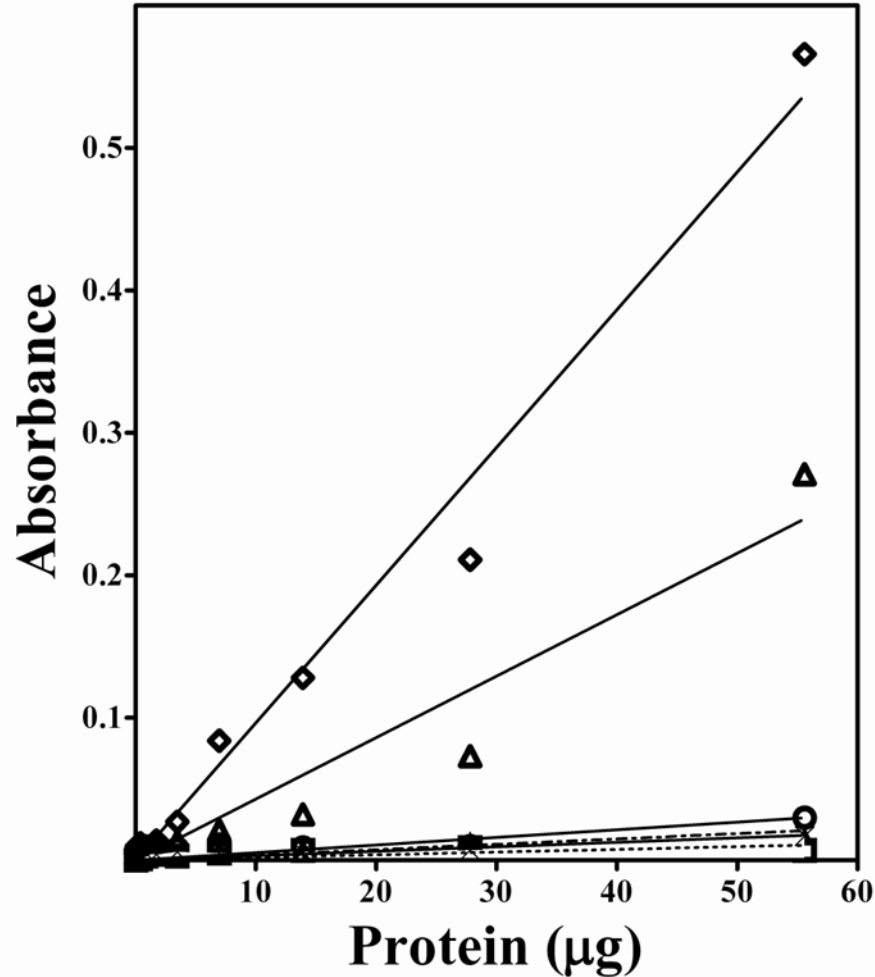


Figure 4.10: **Pik1p and Pik1p^{D709A} bind to Cdc4p, but Pik1p^{R838A} does not.**

Multiwell plates were coated with purified Cdc4p as described in the methods. Wells were subsequently probed with serial dilutions of homogenates from *S. pombe* cells transformed with the pREP1 expression vector controlling the expression of: the full-length *pik1*^{wt} cDNA sequence (triangle); the full-length *pik1* cDNA sequence carrying mutations R838A (circle); D709A (diamond); or R838A and D709A (cross); or the empty vector (square, dotted line). Only cells carrying the *pik1*^{wt} allele were cultured in the presence of thiamine as a negative control (closed triangle). After washing, the wells were probed with a primary antibody against Pik1p and a secondary antibody:HRP conjugate and color development was monitored. Results are expressed as a change in absorbance (OD₆₅₀₋₄₅₀). Results shown are representative of 3 independent experiments.

ectopically expressing the *pik1*^{D709A} allele suggest that the Pik1p^{D709A} protein accumulates to levels similar to the wild-type protein. Thus, in these assay conditions, Pik1p^{D709A} may be interacting with a higher affinity to Cdc4p than the wild-type protein. In either case, the Pik1p^{D709} residue is not required for the interaction with Cdc4p.

No evidence of an interaction between Cdc4p and Pik1p was observed when homogenates of cells carrying expressed Pik1p^{R838A} protein was incubated with the Cdc4p coated multiwell plates. The signal produced by the homogenates of cells carrying the Pik1p^{R838A} protein was similar to background levels over the range of 18-56 µg of homogenate protein (Figure 4.10). Likewise, ELISAs of cell lysates with expressed Pik1p protein containing both the D709A and R838A mutations did not bind to Cdc4p. Thus, the Pik1p^{R838} residue is required for the Cdc4p interaction with full-length Pik1p in ELISAs.

4.7. The chromosomal integration of the *pik1* alleles

4.7.1. Pik1p lipid kinase activity is essential for *S. pombe* cell viability

To evaluate if the lack of *pik1* lipid kinase activity is deleterious to *S. pombe* haploid growth, the *pik1*^{D709A} mutation was integrated into the *pik1* chromosomal locus (see Materials and Methods, Section 3.1.4.2). The *pik1* allelic integration construct is described in Figure 3.1.

Cells with an integrated *pik1*^{wt} sequence were used as a positive control to ensure that the construct was not lethal for haploid growth (Figure 4.11). Diploid cells carrying the *pik1*^{wt}, *nmt1* terminator and *ura4* gene cassette were induced to undergo

A.

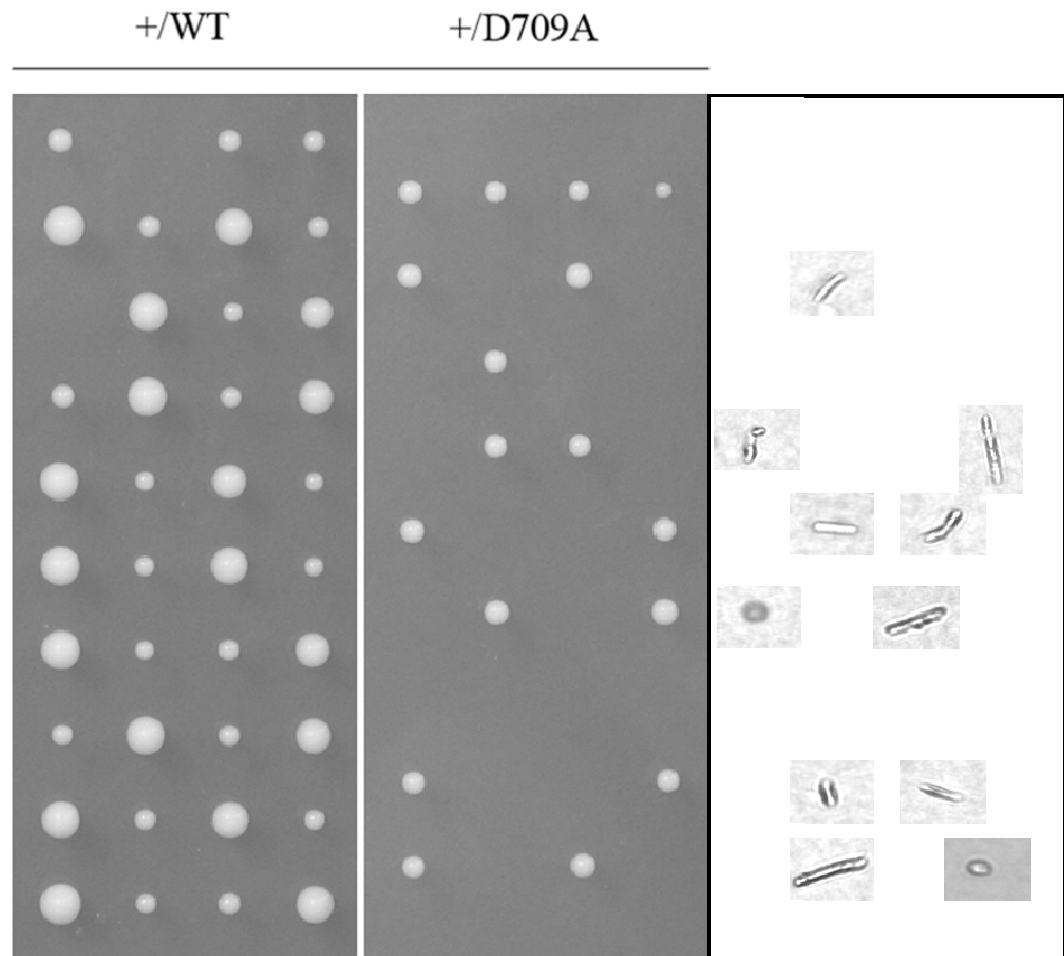
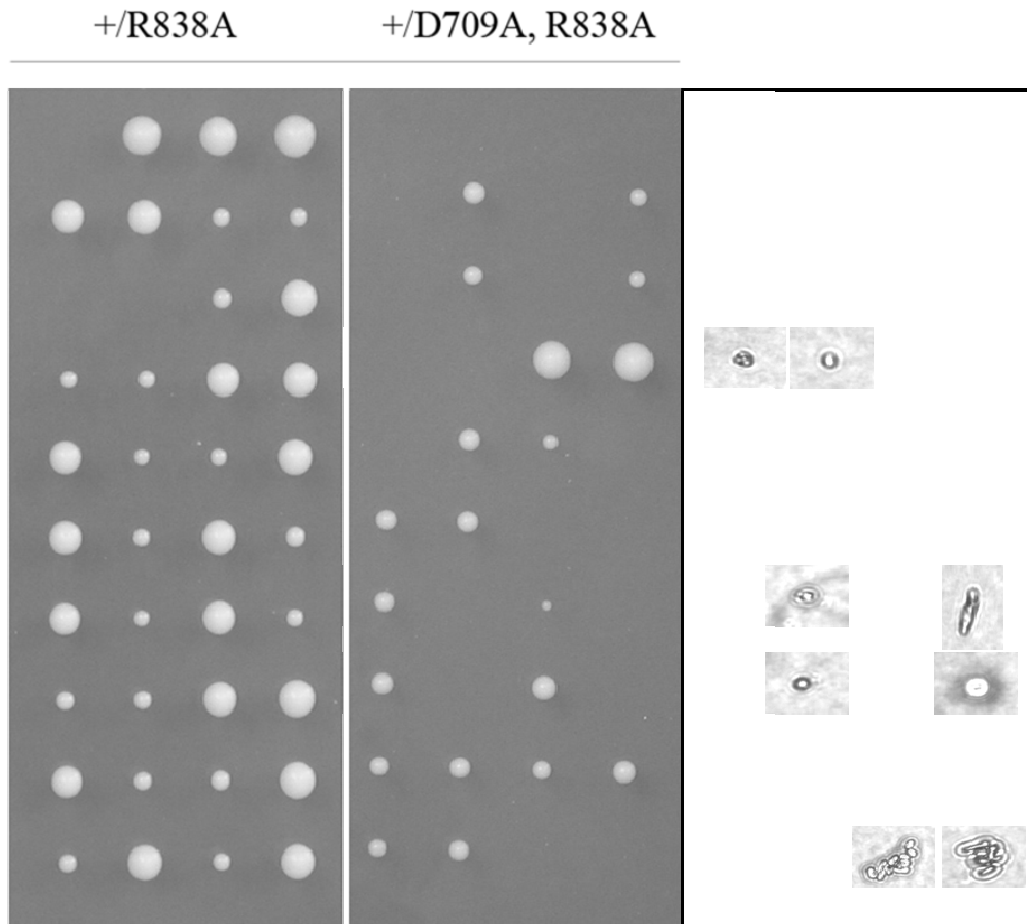


Figure 4.11: Replacement of the genomic wild-type *pik1* sequence with *pik1* mutant alleles.

(A) Constructs were composed of the C-terminal region of the *pik1* allele with or without the D709A mutation, a STOP codon, *nmt1* terminator, *ura4* gene cassette and genomic DNA immediately downstream of the *pik1* coding region. Homologous recombination was used to introduce the *pik1*^{D709A} mutation into the *pik1* chromosomal locus in diploid cells. Diploid cells positive for the integration of the construct into the genome were induced to sporulate on ME plates. The 4 spores from azygotic asci were separated with a micromanipulator on YES. The spores were then allowed to germinate on YES for 4 days at 30°C. Dissected asci designated +/WT indicate that the diploids are heterozygous for the integrated *pik1*^{wt} construct (strain N1550) and dissected asci designated +/D709A indicate that the diploids are heterozygous for the *pik1*^{D709A} construct (strain N1565).

B.



(B) Homologous recombination was used to introduce the *pik1*^{R838A} and *pik1*^{D709A, R838A} mutations into the *pik1* chromosomal locus in diploid cells. Constructs were constructed as described in A. Diploid cells positive for the integration of the construct into the genome were induced to sporulate on ME plates. The 4 spores from azygotic asci were separated and grown as described in A. The +/R838A designation indicates that these asci were heterozygous for the *pik1*^{R838A} mutation and the *pik1* wild-type genomic locus. The +/D709A, R838A designation indicates that these asci were heterozygous for the *pik1*^{D709A, R838A} mutations and the *pik1* wild-type genomic locus.

meiosis and sporulation on ME plates. A Zeiss tetrad micromanipulator was then used to dissect out spores from azygotic asci on YES plates. As expected, the 4 spores pulled from 8 out of 10 asci formed colonies at 30°C (Figure 4.11A). Surprisingly however, two out of the 4 spores pulled from each ascus formed colonies more rapidly. Colony formation on plates lacking uracil and colony PCR revealed that the faster growing colonies had the integrated construct (not shown). It is uncertain why this is the case since each spore is expressing the *pik1*^{wt} sequence under its native promoter. However, it is possible that the inclusion of the *nmt1* terminator affects *pik1* translation causing cells to grow at a different rate. Two asci showed a 3:1 segregation, with three spores forming colonies and one spore not forming a colony (Figure 4.11A). Again, it is unclear why this would happen, but it was likely that the single spore failed to germinate due to unknown reasons.

To establish whether Pik1p lipid kinase activity was essential for *S. pombe* cell viability, the *pik1*^{D709A} mutation was integrated into the genome at the *pik1* chromosomal locus. Diploids were allowed to enter meiosis and tetrad analysis was performed. If lipid kinase activity is essential for cell viability, one would expect only half the spores to form viable colonies since the diploids are heterozygous for the *pik1*^{D709A} mutation. Thus, two spores should contain wild-type *pik1* and two spores should contain the *pik1*^{D709A} mutation. Tetrad analysis revealed that 6 out of 10 asci produced a 2:2 segregation of viable to non-viable colonies, with two spores from each ascus forming colonies and two spores not forming colonies (Figure 4.11A). Cells from the growing colonies were then streaked out on plates lacking uracil. The cells did not grow on these plates indicating that they lacked the *ura4* gene cassette and did not carry

the integration construct carrying the *pik1*^{D709A} mutation. One ascus out of 8 produced 4 colony forming spores (Figure 4.11A). Cells from these colonies were streaked onto EMM plates lacking uracil to assess for the presence of the *ura4* gene cassette. The cells from all 4 spores did not grow on EMM –Ura plates indicating that each spore did not carry the integration construct with the *ura4* gene cassette (data not shown). This was confirmed by colony PCR. Dissection of one asci out of 8 also produced one colony forming spore (Figure 4.11A). This was likely the case of a 2:2 segregation, but for some unknown reason, the second spore did not form a colony. Colony formation on plates lacking uracil and colony PCR demonstrated that the viable colony did not have the integrated allele and was wild-type (data not shown). Microscopic examination of the spores that failed to form colonies indicated that the majority of the cells germinated, but failed to divide (Figure 4.11A). Many cells were also elongated suggesting a cytokinesis defect. Thus, Pik1p lipid kinase activity appears to be essential for *S. pombe* cell viability.

4.7.2. The *pik1* R838 residue is not essential for cell viability in *S. pombe*

The results presented thus far suggest that the *pik1* residue R838 is required for the Cdc4p-Pik1p interaction. Therefore, it was of interest to evaluate the expression of the *pik1*^{R838A} allele under the control of its native promoter. To this aim, the *pik1*^{R838A} allele replaced the wild-type genomic *pik1* locus of *S. pombe* wild-type diploids as described previously for the wild-type and D709A versions of *pik1*. Diploid cells positive for the integrated construct were induced to sporulate on ME plates and the tetrads isolated and dissected with a micromanipulator. All 4 spores isolated from 8 out

of 10 asci heterozygous for the *pik1*^{R838A} mutation formed colonies on YES plates (Figure 4.11B). Like the colonies with the integrated wild-type *pik1-ura4* construct, two out of the four colonies which formed from spores from a single ascus appeared larger than the other two, demonstrating a 2:2 segregation of cells expressing *pik1*^{R838A} with cells wild-type for genomic *pik1* (Figure 4.11B). In two out of the 8 asci, there were only 2 or 3 colonies that formed from a tetrad for unknown reasons (Figure 4.11B). The colonies that grew from spores isolated from the tetrad were streaked onto media lacking uracil to validate the presence of the integrated *pik1*^{R838A} construct. It was shown that the larger colonies were *ura*⁺ indicating that they contained the inserted DNA. These results were very similar to the integrated wild-type sequence shown previously. The insertion of the *pik1*^{R838A} mutation was confirmed by colony PCR and sequencing.

The double-mutant version of the *pik1* allele carrying both the D709A and R838A mutations (*pik1*^{D709A, R838A}) was also integrated into the genome at the *pik1* locus. As expected, since this *pik1* allele lacked lipid kinase activity, tetrad analysis of 8 out of 10 asci pulled showed a 2:2 viable to non-viable phenotype, with 2 spores from each ascus forming colonies and 2 spores from each ascus not forming colonies (Figure 4.11B). One ascus produced 4 viable colonies (Figure 4.11B). These colonies were streaked onto plates lacking uracil and all 4 spores were not uracil prototrophs indicating that the ascus was a wild-type contaminant (data not shown). Examination of the spores that failed to form colonies indicated that some spores failed to germinate (Figure 4.11B). One spore grew into a cell that failed to divide, and two spores formed microcolonies. Thus, variable phenotypes were observed. It is unclear whether the

R838A mutation caused the spores to fail to germinate in combination with the D709A mutation.

In summary, *pik1* lipid kinase activity is essential for *S. pombe* cell viability, whereas *pik1* Cdc4p-binding activity is not essential for *S. pombe* cell viability.

4.8. The ectopic expression of the *pik1* alleles

4.8.1. Ectopic expression of *pik1*^{wt} and mutant alleles were used to assess the importance of disrupting *pik1* lipid kinase activity and Cdc4p binding activity on cell growth and division

The ectopic expression of the wild-type and mutant alleles of a gene is a common approach for evaluating protein function in cells. In these experiments, the ectopic expression of the wild-type and 3 mutant *pik1* alleles were analyzed separately in *S. pombe* cells and in parallel. Ectopic expression was performed in cells with an intact chromosomal copy of *pik1*.

4.8.2. Ectopic expression of *pik1*^{wt} in *S. pombe* cells impairs colony formation

The *pik1*^{D709A} mutation abolished, or greatly reduced *pik1* lipid kinase activity. Expression of the *pik1*^{D709A} mutation under the control of its native promoter, through integration of the D709A mutation into the *pik1* chromosomal locus, is lethal to *S. pombe* cells. To evaluate Pik1p protein function, ectopic expression of the *pik1*^{wt} and *pik1*^{D709A} alleles from the full-strength *nmt1* promoter was performed. First, *pik1*^{wt} was ectopically expressed in wild-type *S. pombe* cells. Cells carrying the pREP1 *pik1*^{wt} episome were grown to stationary phase (7-9 x 10⁷ cells/mL) for 24-48 hours at 30°C in

the presence of thiamine. The cells were washed free of thiamine, and resuspended in their original volume with water. The cells were then serially diluted from 10^7 to 10^3 cells/mL. Aliquots (5 μ L) were spotted onto EMM lacking leucine plates supplemented with phloxin B. Phloxin B is a viability stain that stains dead cells red. As a negative control, cells carrying the vector alone were cultured in parallel. Cells were spotted onto plates with or without thiamine to evaluate the effects of *pikI*^{wt} gene expression under the control of the *nmtI* promoter. Cells carrying the vector alone formed colonies that were pink/white up to the highest dilution (Figure 4.12). The pink/white color indicates that the colonies contained mainly viable cells. The cells formed colonies equally well irrespective of thiamine (Figure 4.12). Cells carrying the *pikI*^{wt} allele grown on plates containing thiamine formed colonies indistinguishably from cells carrying the vector alone (Figure 4.12). In contrast, cells carrying the *pikI*^{wt} allele spotted on plates lacking thiamine demonstrated a profound effect on colony formation. Colony formation was markedly impaired and the colonies were red (Figure 4.12). Thus, ectopic expression of *pikI*^{wt} is lethal to *S. pombe* cells. Colony formation of cells incubated at 19°C, 25°C and 37°C did not indicate any temperature dependent effects.

The growth parameter of cells is better established in liquid cultures. A 50 mL culture of cells was started at a cell density of 1×10^5 cells/mL and grown in the presence and absence of thiamine at 30°C. The rate of cell proliferation was then followed for 48 hours. Cells carrying the *pikI*^{wt} allele grown in repressed conditions grew very similarly to cells carrying the vector alone grown in repressed and derepressed conditions (Figure 4.13). This was in contrast to cells ectopically expressing the *pikI* wild-type allele which ceased to divide after 24 hours (Figure 4.13). The effects of the

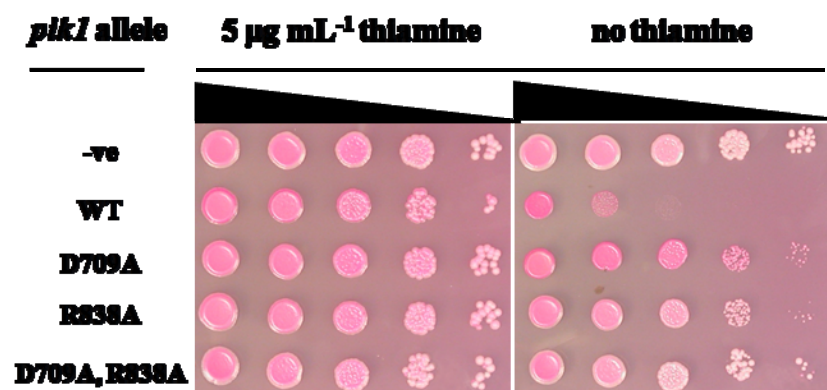


Figure 4.12: Ectopic expression of *pik1* and mutant alleles affect colony formation in *S. pombe*.

Serial dilutions of cells transformed with the pREP1 expression vector containing: no insert (-ve); *pik1* wild-type (WT); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A) were spotted onto EMM –Leu plates that contained phloxin B. Aliquots (5 μL) of each dilution of 10^5 to 10^1 cells/mL were applied per spot (ramp). Culture medium was supplemented with thiamine or not as indicated and plates were incubated at 30°C for 6 days. Results shown are representative of 3 independent experiments.

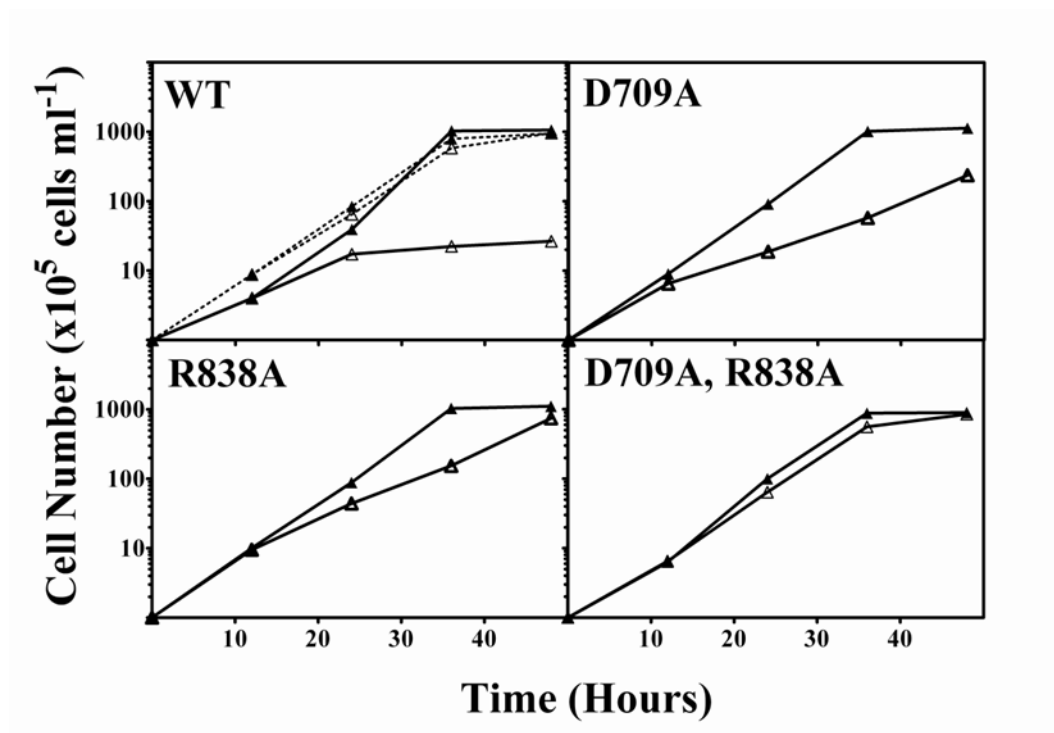


Figure 4.13: Ectopic expression of *pik1* alleles affects cell proliferation.

Cells were transformed with pREP1 expression vector containing: no insert (WT, dotted lines); *pik1* wild-type (WT, solid lines); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A) sequences. Cells transformed with the vector alone (no insert) and cultured in the presence and absence of thiamine are shown as dotted lines. Cell cultures were started at a cell density of 1×10^5 cells/mL in EMM –Leu and incubated at 30°C in the presence (closed triangle) or absence (open triangle) of thiamine. Results shown are from a single culture.

ectopic expression of the *pik1* wild-type allele on cell proliferation were dosage dependent since cell proliferation was mildly or not affected when *pik1^{wt}* gene expression was derepressed under the control of the attenuated and highly attenuated *nmt1* promoters of the pREP41 and pREP81 vectors (Figure 4.14A and B).

Since cells ectopically expressing the *pik1^{wt}* allele failed to form colonies at the 10^5 to 10^3 dilutions, and failed to proliferate after 24 hours, the ectopic expression of *pik1^{wt}* was likely lethal. Cell viability assays however, were not performed. The viability stain phloxin B was used in this study, and the colonies which formed at the 10^7 and 10^6 dilutions after the ectopic expression of *pik1^{wt}* were red. Dead cells have increased permeability to phloxin B suggesting that these cells were indeed inviable. Thus, ectopic expression of *pik1^{wt}* appears to be lethal in wild-type *S. pombe* cells.

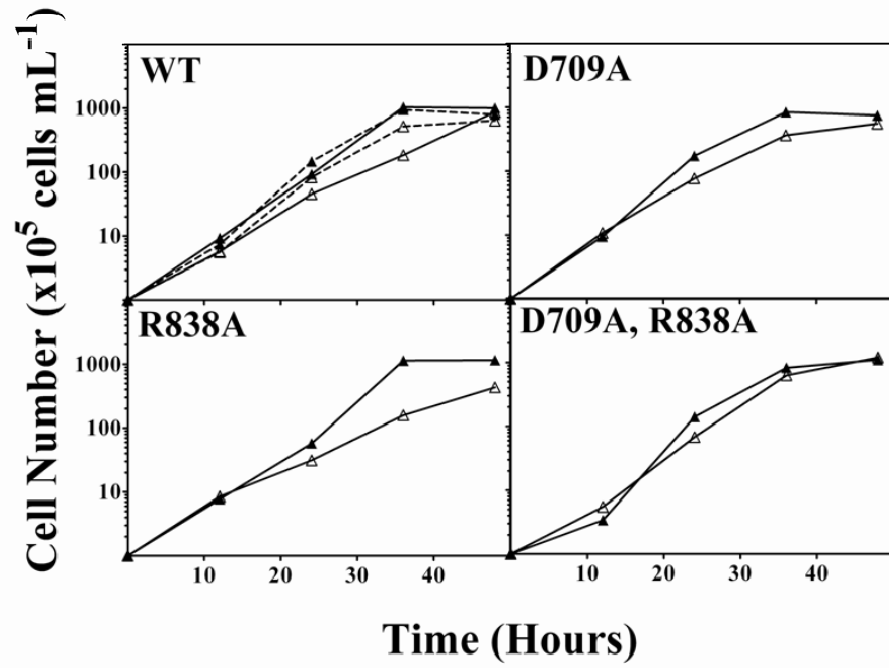
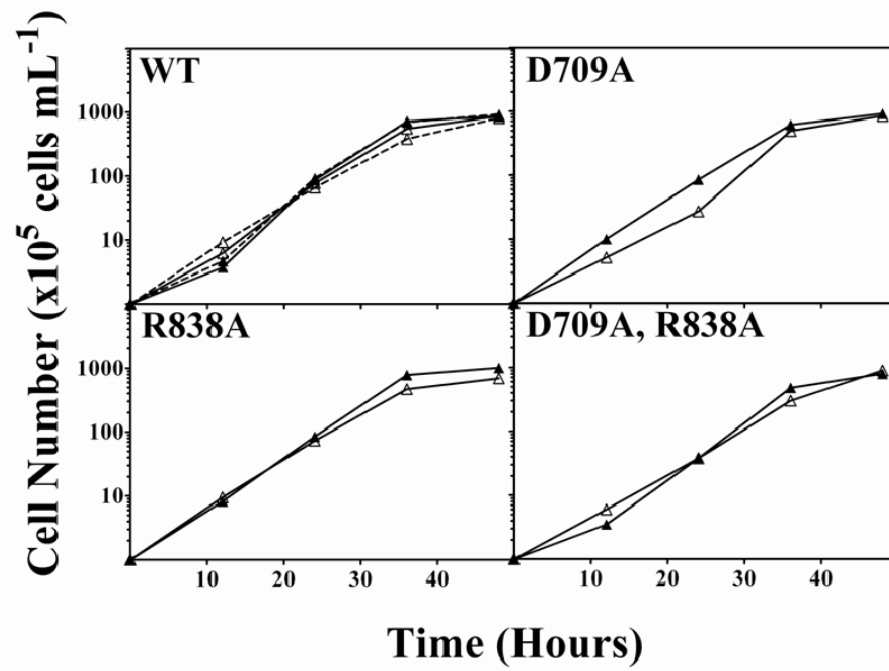
4.8.3. Ectopic expression of the *pik1^{D709A}* allele does not impair cell proliferation to the same extent as the ectopic expression of the wild-type enzyme

On plates containing thiamine, cells carrying the *pik1^{D709A}* allele formed colonies indistinguishably from cells carrying the vector alone on plates containing or lacking thiamine (Figure 4.12). Cells carrying the *pik1^{D709A}* allele grown in derepressed conditions were able to form colonies to a greater extent than cells carrying the wild-type *pik1* allele (Figure 4.12). This result suggests that the ectopic expression of *pik1^{D709A}*, which has no lipid kinase activity, is not as lethal as the ectopic expression of the catalytically active enzyme.

In liquid culture, cells carrying the *pik1^{D709A}* allele grown in repressed conditions proliferated at similar rates to cells carrying the vector alone (Figure 4.13).

Figure 4.14: Cell proliferation is mildly or not affected upon the ectopic expression of *pik1* alleles under the control of the attenuated and highly attenuated *nmt1* promoters of the pREP41 and pREP81 plasmids.

Cells were transformed with pREP41 (A) and pREP81 (B) expression vectors containing: no insert (WT, dotted lines); *pik1* wild-type (WT, solid lines); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A) sequences. Cells transformed with the vector alone (no insert) and cultured in the presence and absence of thiamine are shown as dotted lines. Cell cultures were started at a cell density of 1×10^5 cells/mL in EMM –Leu and incubated at 30°C in the presence (closed triangle) or absence (open triangle) of thiamine. Results shown are from a single culture.

A.**B.**

However, when cells were ectopically expressing the *pikI*^{D709A} allele, cell proliferation became slower, but the cells continued to proliferate over the 48 hour period (Figure 4.13). This is in marked contrast to the ectopic expression of the wild-type sequence which caused cells to cease dividing after 24 hours. The effects of the ectopic expression of the *pikI*^{D709A} allele were dosage dependent because ectopic expression from the pREP41 and 81 vectors had no effect on cell proliferation in liquid culture (Figure 4.14). Thus, in accord with the colony formation assays, ectopic expression of the *pikI*^{D709A} allele in liquid culture did not impair cell proliferation to the same extent as the *pikI* wild-type allele.

4.8.4. Ectopic expression of *pikI*^{wt} disrupts actin cytoskeletal structures

There were no obvious differences in cell morphology upon the ectopic expression of the *pikI* wild-type sequence compared to cells carrying the vector alone under bright field microscopy (data not shown). The length of cells carrying the *pikI* wild-type sequence ranged from 6-17 µm and the results were similar when cells were grown in the presence or absence of thiamine (Figure 4.15). The length of the cells was also similar to that of cells carrying the vector alone grown in the presence and absence of thiamine (Figure 4.15). The mean length of cells ectopically expressing the *pikI* wild-type sequence was slightly reduced compared to cells cultured in repressed conditions or cells carrying the vector alone grown in repressed and derepressed conditions (Table 4.2). Similar results were observed upon the ectopic expression of *pikI*^{wt} under the control of the attenuated *nmtI* promoter of the pREP41 plasmid (data not shown).

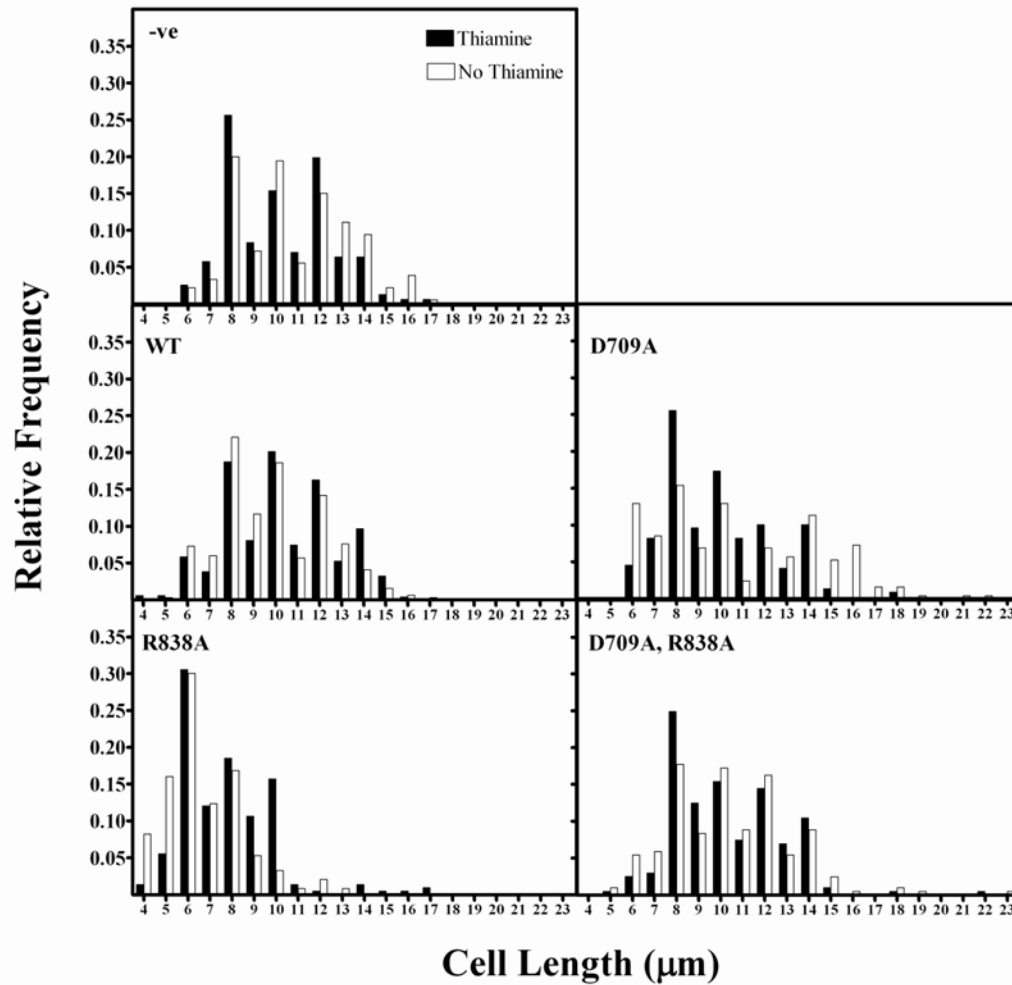


Figure 4.15: **Histograms of cell length of *S. pombe* wild-type cells after ectopic expression of *pik1* alleles.**

S. pombe wild-type cells transformed with the pREP1 expression vector containing: no insert (-ve), *pik1* wild-type (WT); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A). Cell length was measured from a population of cells grown for 24 hours at 30°C in the presence (black bars) or absence (white bars) of thiamine. Cell length was measured from photographs of cells stained with CF+DAPI relative to a micrometer bar. Between 150-300 cells were measured for each allele cultured in the presence or absence of thiamine from a single culture.

Table 4.2.

Effects of ectopic expression of *pik1* alleles on mean cell length (μm) of wild-type cells

Allele	+ thiamine	no thiamine
-	10.1 ± 0.2	10.7 ± 0.2
<i>pik1</i>	10.2 ± 0.1	9.8 ± 0.3
<i>pik1</i> ^{D709A}	9.8 ± 0.2	10.6 ± 0.2
<i>pik1</i> ^{R838A}	7.6 ± 0.1	6.4 ± 0.1
<i>pik1</i> ^{D709A,R838A}	10.2 ± 0.2	10.3 ± 0.2

Cell cultures were at 30°C for 24 hours with or without thiamine. Cell length was measured from a population of cells grown for 24 hours at 30°C in the presence or absence of thiamine. Cell length (in μm) was measured from photographs of cells stained with CF+DAPI relative to a micrometer bar. Results shown are mean \pm S.E. of 150-300 cells measured for each allele from a single culture.

As cells ectopically expressing the *pikI^{wt}* allele cease to proliferate after 24 hours, it was of interest to examine the ability of these cells to form contractile rings and septa. Cells were grown for 24 hours at 30°C in the presence or absence of thiamine, formaldehyde fixed and stained with calcofluor white (CF) and DAPI. CF stains the septum and DAPI stains DNA, acting as a marker for the nucleus. Cells carrying the vector alone cultured in the presence and absence of thiamine showed staining with CF as a medial band in ~10% of the cell population (Table 4.3). Accordingly, ~10% of the cells were binucleated. The same was observed in cells carrying the *pikI* wild-type sequence grown in repressed and derepressed conditions (Table 4.3). However, cells ectopically expressing the *pikI^{wt}* sequence exhibited a more intense medial CF staining (Figure 4.16).

Cells carrying the vector alone and the *pikI^{wt}* allele were grown for 24 hours at 30°C in the presence and absence of thiamine, formaldehyde fixed and stained with FITC-phalloidin to observe actin cytoskeletal structures. Cells carrying the vector alone showed the localization of actin to patches at the tips of cells and to the medial ring (Figure 4.16). The same actin distributions were observed in cells carrying the vector alone irrespective of the presence of thiamine and were also observed in cells carrying the *pikI^{wt}* allele grown in the presence of thiamine (not shown). The proportion of the cell population showing FITC-phalloidin staining at the medial region was approximately 10% (Table 4.3). However, when the cells were expressing the *pikI^{wt}* allele, F-actin was no longer localized to the cell tips or to the medial region. Instead, bright punctate staining throughout the cell was observed (Figure 4.16). Only 1% of these cells had F-actin staining at the medial region (Table 4.3). F-actin associates with

Table 4.3.

Effects of ectopic expression of *pik1* alleles on characteristics of cytokinesis in *S. pombe* cells

	+ thiamine				no thiamine			
	CR ^{Actin}	CR ^{Myosin}	Septa	BiN	CR ^{Actin}	CR ^{Myosin}	Septa	BiN
	(frequency, %)				(frequency, %)			
-	8	10	9	6	10	9	9	9
WT	9	9	9	10	1	1	10	10
D709A	8	9	10	11	8	8	20	29
R838A	14	8	9	10	14	8	10	13
D709A, R838A	8	8	8	10	10	8	7	10

Cell cultures were at 30°C for 24 hours with or without thiamine. Cells were evaluated for the presence of the contractile ring (CR, visualized with FITC-phalloidin for F-actin and indirect immunofluorescence for myosin II, Myo2p), septa (visualized with calcofluor-white) and two nuclei (BiN with DAPI). Results are expressed as % of the cell population examined with n=100 cells for each culture.

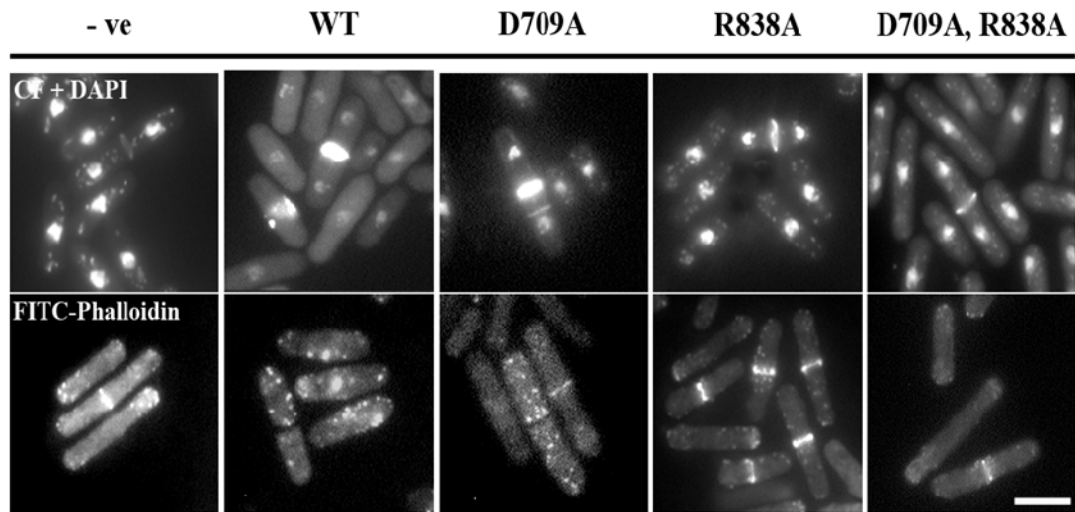


Figure 4.16: Septum formation and F-actin distribution in cells after the ectopic expression of *pik1* alleles.

S. pombe wild-type cells transformed with the pREP1 expression vector containing: no insert (-ve); *pik1* wild-type (WT); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A). Cells shown were grown for 24 hours at 30°C in the absence of thiamine and fixed with formaldehyde. Thiamine inhibits expression from the *nmr1* promoter. Cells were examined by epifluorescence microscopy after staining with calcofluor white plus DAPI, or FITC-phalloidin. Bar=5 µm. Results shown are representative of 2 independent experiments.

the type II myosin Myo2p at the contractile ring. In support of the FITC-phalloidin staining, a 10-fold reduction in the number of cells with anti-Myo2p medial bands was observed compared to cells carrying the vector alone or cultured in the presence of thiamine (Table 4.3).

Localization of the ectopically expressed *pik1^{wt}* protein was then examined by indirect immunofluorescence with anti-Pik1p polyclonal antiserum. Cells were grown for 24 hours at 30°C, methanol fixed and examined using a preimmune serum and a polyclonal antiserum generated against Pik1p. The secondary antibody was an anti-rabbit IgG antibody conjugated to Texas Red. With preimmune antiserum, there was no significant localized fluorescence in cells expressing *pik1^{wt}* (Figure 4.17). In cells transformed with the vector alone, faint punctate fluorescence could be observed likely reflecting the endogenous *pik1^{wt}* localization (Figure 4.17). The same was observed in cells carrying the wild-type *pik1* sequence grown under repressed conditions (data not shown). However, after 24 hours of growth in liquid media lacking thiamine, a marked increase in Pik1p immunofluorescent intensity was observed throughout the cells (Figure 4.17). When *pik1^{wt}* was expressed under the attenuated and highly attenuated *nmt1* promoters of the pREP41 and pREP81 plasmids, the intensity of fluorescence was greatly reduced compared to that observed upon the expression of *pik1^{wt}* under the full-strength *nmt1* promoter (data not shown). There is apparent variability in immunostaining observed between cells ectopically expressing the *pik1^{wt}* allele, with some cells showing intense staining and others much less intense staining. This may be due to stochastic gene expression and/or differences in the process of immunostaining. Controls such as immunostaining with the secondary antibody alone (goat IgG

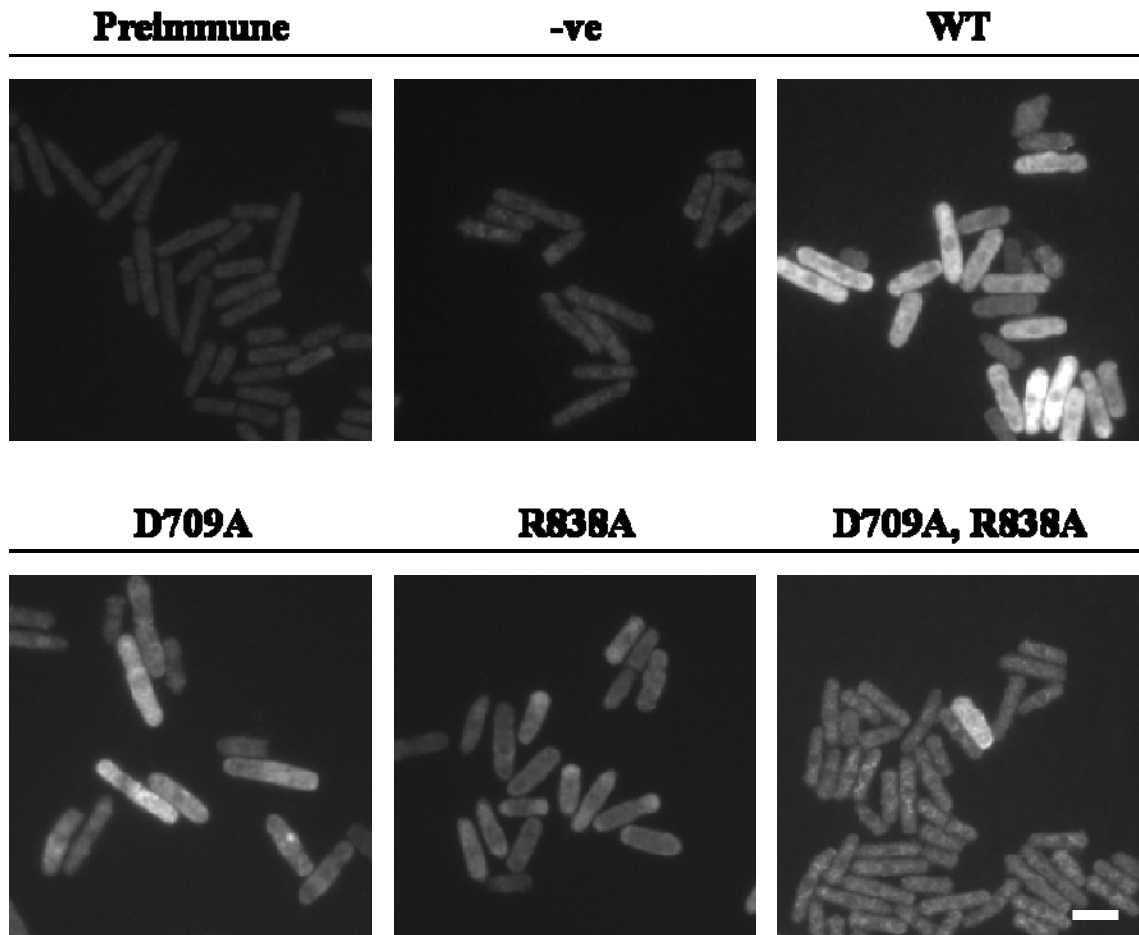


Figure 4.17: α -Pik1p immunostaining after ectopic expression of *pik1* alleles.

S. pombe wild-type cells transformed with the pREP1 expression vector containing: no insert (-ve); *pik1* wild-type (WT); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A). Cells shown were grown for 24 hours at 30°C in the absence of thiamine and fixed with methanol. Cells were examined by indirect immunofluorescence microscopy after incubating the cells with a primary rabbit antiserum against Pik1p and a secondary antibody against rabbit IgG conjugated to Texas Red. Bar=5 μ m. Results shown are representative of 2 independent experiments. Controls such as immunostaining with the secondary antibody alone (goat IgG conjugated to Texas Red) and cells grown in the presence of thiamine were also immunostained.

conjugated to Texas Red) and cells grown in the presence of thiamine were also immunostained.

In summary, upon the removal of thiamine, the Pik1p protein accumulates in *S. pombe* cells and is catalytically active. Cell proliferation ceases after 24 hours, presumably due to the disruption of F-actin structures, including the formation of the contractile ring.

4.8.5. Ectopic expression of the *pik1*^{D709A} allele impairs septation

Ectopic expression of the *pik1*^{D709A} allele reduced cell proliferation, but not to the same extent as the ectopic expression of the wild-type sequence. The cell appearance under bright field microscopy did not differ between cells ectopically expressing the *pik1*^{D709A} allele and cells carrying the vector alone cultured in repressed or derepressed conditions (data not shown). However, the length of cells ectopically expressing the *pik1*^{D709A} allele ranged from 6-22 μm (Figure 4.15). About 15-20% of these cells were elongated ($>15 \mu\text{m}$ in length) relative to cells cultured under repressed conditions or expressing the *pik1*^{wt} sequence (Figure 4.15). Accordingly, the mean cell length increased from $9.8 \pm 0.2 \mu\text{m}$ in repressed conditions to $10.6 \pm 0.2 \mu\text{m}$ in derepressed conditions (Table 4.2).

Cells carrying the pREP1 *pik1*^{D709A} allele cultured in the presence of thiamine showed septum staining indistinguishable from cells carrying the vector alone cultured in media containing or lacking thiamine (data not shown). However, when cells carrying the *pik1*^{D709A} allele were cultured in media lacking thiamine, a doubling in the number of cells with septa was observed ($\sim 20\%$ from $\sim 10\%$ in the presence of

thiamine) (Table 4.3). A proportion of these septated cells (~6%) had more intense CF staining or more than one septa. This was not observed in control cells carrying the vector alone. Cells carrying the *pik1*^{D709A} allele cultured in the presence of thiamine demonstrated F-actin staining indistinguishable from control cells carrying the vector alone (data not shown). However, in some of the cells ectopically expressing the *pik1*^{D709A} allele, F-actin was no longer observed in patches at the tips of cell ends, but found dispersed throughout the cell (Figure 4.16). The number of cells with actin rings however was comparable to cells carrying the vector alone or cultured under repressed conditions (~10%) (Table 4.3). Immunostaining with anti-Myo2p antibodies confirmed these results (data not shown). The Pik1p indirect immunofluorescent distribution in cells ectopically expressing the *pik1*^{D709A} allele was similar to that observed in cells ectopically expressing the *pik1*^{wt} allele (Figure 4.17).

In summary, derepression of *pik1*^{D709A} expression results in the accumulation of Pik1p^{D709A} protein, which has no lipid kinase activity, but binds to Cdc4p. Cell proliferation is reduced, but not to the same extent as the ectopic expression of the *pik1* wild-type sequence. Many cells are elongated with thick or multiple septa. These results suggest a possible role for *pik1* lipid kinase activity in septation.

4.8.6. Residue R838 of *pik1* is required for *pik1*^{wt} ectopic lethality

Ectopic expression of *pik1*^{wt} was lethal likely due to disrupted actin cytoskeletal structures including the formation of the contractile ring (Section 4.8.2). Ectopic expression of a kinase-dead allele of *pik1* did not have the same effect, but caused septation defects. It was shown that the *pik1*^{R838} residue was required for the

interaction with Cdc4p in yeast two-hybrid assays and ELISAs. Therefore, it was of interest to study the effects of the ectopic expression of the *pikI*^{R838A} allele on cell proliferation and morphology. For the colony formation assay, as described previously, cells carrying the pREP1 *pikI*^{R838A} plasmid were grown to 7-9 x10⁷ cells/mL at 30°C, serially diluted 10-fold five times and 5 µL spotted onto plates containing or lacking thiamine.

Cells carrying the *pikI*^{R838A} allele and grown in repressed conditions formed colonies indistinguishably from cells carrying the vector alone (Figure 4.12). In derepressed conditions, cells carrying the *pikI*^{R838A} allele formed pink/white colonies that were slightly smaller than the colonies carrying the vector alone, or cells carrying the *pikI*^{R838A} allele in repressed conditions (Figure 4.12). The colonies ectopically expressing the *pikI*^{R838A} allele however were pink/white in color suggesting that the cells maintained membrane integrity and were excluding phloxin B. This was in marked contrast to cells ectopically expressing the *pikI* wild-type sequence which severely impaired colony formation and increased permeability to phloxin B.

In liquid cultures, cells carrying the *pikI*^{R838A} allele grown in repressed conditions proliferated at similar rates to cells carrying the vector alone (Figure 4.13). Upon the ectopic expression of the *pikI*^{R838A} allele only a small decline in cell proliferation was observed (Figure 4.13). The rate of cell proliferation of cells ectopically expressing the *pikI*^{R838A} allele under the attenuated *nmtI* promoter of the pREP41 plasmid was similar to that of *pikI*^{R838A} expression under the full-strength *nmtI* promoter (Figure 4.14). No effect on cell proliferation was observed when the *pikI*^{R838A}

sequence was ectopically expressed under the highly attenuated pREP81 *nmtI* promoter (Figure 4.14).

Cells carrying the pREP1 *pikI*^{R838A} plasmid cultured for 24 hours at 30°C in the absence of thiamine were much shorter in length compared to cells carrying the vector alone or *pikI*^{wt} sequence cultured under similar conditions (Table 4.2). Even under repressed conditions, cells carrying the *pikI*^{R838A} allele were shorter compared to cells carrying the vector alone grown in repressed and derepressed conditions (Table 4.2). The length of cells ectopically expressing the *pikI*^{R838A} allele was reduced to a range of 4-11 µm compared to cells carrying the vector alone which had a cell length distribution of 6-16 µm (Figure 4.15). In the absence of thiamine, there were more cells in the range of 4-6 µm than in cells cultured in the presence of thiamine (Figure 4.15).

Although the cells were shorter, the proportion of the cell population with a septum, as visualized with CF staining, and were binucleated, was comparable to cells carrying the vector alone or cells carrying the *pikI*^{R838A} allele in repressed conditions (Table 4.3; Figure 4.16). The intensity of CF staining was also comparable to cells carrying the vector alone (Figure 4.16).

Cells carrying the *pikI*^{R838A} allele grown in the presence of thiamine had F-actin cytoskeletal structures, as visualized with FITC-phalloidin, indistinguishable from cells carrying the vector alone cultured in the presence and absence of thiamine (data not shown). Under derepressed conditions, cells ectopically expressing the *pikI*^{R838A} allele had F-actin patches at the tips of cells similar to that observed in cells carrying the vector alone (Figure 4.16). The relative number of pREP1 *pikI*^{R838A} cells with F-actin staining at the medial region was unaffected by the presence or absence of thiamine

(Table 4.3). The ectopic expression of the *pikI*^{R838A} allele had no apparent effect on contractile ring formation in contrast to the ectopic expression of the *pikI*^{wt} sequence which resulted in a 10-fold reduction in the number of cells with contractile rings (Table 4.3). Immunostaining with Myo2p to observe contractile ring structures, confirmed the results obtained with F-actin staining (Table 4.3).

Interestingly, upon ectopic expression of the *pikI*^{R838A} allele, localization of the Pik1p protein appeared to be concentrated at the cell tips of many of the shorter cells (Figure 4.17). This is distinct from the localization of the *pikI*^{wt} and *pikI*^{D709A} alleles which appeared to be evenly distributed throughout the cell upon their ectopic expression. This result suggests that the Pik1p R838A mutation may affect the localization of the ectopically expressed Pik1p protein. In contrast, expression of *pikI*^{R838A} under the control of the attenuated *nmtI* promoter of the pREP41 plasmid had little to no effect on contractile ring formation, actin patches and septa (data not shown). Indirect immunofluorescence with α -Pik1p showed decreased fluorescence relative to ectopically expressed *pikI*^{R838A} under the full-strength *nmtI* promoter (data not shown). Staining at the cell tips was also not observed likely due to the much lower level of expression from the attenuated *nmtI* promoter of the pREP41 plasmid.

In summary, derepression of *pikI*^{R838A} expression caused the accumulation of the protein in the cells, particularly at the cell tips, and this enzyme was catalytically active. Cell proliferation was slightly reduced, and the cells were shorter. Contractile ring formation, F-actin patches at the cell tips, and septa were unaffected. These results clearly indicate a role for the *pikI*^{R838} residue in contributing to the *pikI*^{wt} ectopic lethal phenotype.

4.8.7. Ectopic expression of the *pikI*^{D709A, R838A} mutant has no observable effect on cell proliferation and morphology

The ectopic lethality of the *pikI*^{wt} allele was greatly reduced when the *pikI*^{R838A} allele was expressed instead of the *pikI*^{wt} allele. In comparison, *pikI*^{wt} ectopic lethality was only slightly reduced when the *pikI*^{D709A} allele was expressed instead of the *pikI*^{wt} allele. Therefore, it was of interest to determine if both these residues could fully account for the *pikI*^{wt} ectopic phenotype. In the presence of thiamine, cells carrying the *pikI*^{D709A, R838A} allele formed colonies indistinguishably from cells carrying the vector alone grown in the presence or absence of thiamine (Figure 4.12). In derepressed conditions, cells ectopically expressing the *pikI*^{D709A, R838A} allele also formed colonies indistinguishably from cells grown in repressed conditions, or cells carrying the vector alone (Figure 4.12). In liquid cultures, ectopic expression of the *pikI*^{D709A, R838A} allele had no effect on cell proliferation (Figure 4.13).

The mean length of cells carrying the *pikI*^{D709A, R838A} allele grown in the presence or absence of thiamine was unaffected and similar to cells carrying the vector alone (Table 4.2). The cell length distribution ranged from 6-15 µm in cells ectopically expressing the *pikI*^{D709A, R838A} allele which was comparable to the cell length distribution of cells carrying the vector alone or cells cultured in the presence of thiamine (Figure 4.15).

Cells ectopically expressing the *pikI*^{D709A, R838A} allele had septum staining indistinguishable from cells carrying the vector alone grown in the presence or absence of thiamine, or cells carrying the *pikI*^{D709A, R838A} allele grown in the presence of thiamine (Figure 4.16). Cells carrying the *pikI*^{D709A, R838A} allele grown in the absence of thiamine

showed F-actin cytoskeletal structures similar to cells carrying the vector alone, or cultured in the presence of thiamine such as localization of actin patches to the tips of cells and rings at the medial region (Figure 4.16, Table 4.3). The proportion of the cell population with contractile rings, septa and binucleated cells was unaffected by the ectopic expression of *pik1*^{D709A, R838A} in an asynchronous culture (Table 4.3).

In the absence of thiamine, the *pik1*^{D709A, R838A} protein accumulated in the cells as shown by increased fluorescence relative to cells carrying the vector alone or cells carrying the *pik1*^{D709A, R838A} allele cultured in repressed conditions (Figure 4.17). The *pik1*^{D709A, R838A} protein appears to be evenly distributed throughout the cell, similar to the pattern of accumulation of the wild-type and D709A *pik1* alleles. These results suggest that both the D709 and R838 residues contribute fully to the *pik1*^{wt} ectopic lethal phenotype. In summary, the Pik1p^{D709A, R838A} protein, which has no lipid kinase activity and no Cdc4p-binding activity, accumulates in the cells cultured in the absence of thiamine, but has no effect on cell proliferation or morphology.

4.8.8. The *pik1*^{wt} ectopic phenotype is suppressed in *cdc4*^{G107S} cells

The evidence presented above suggests that the R838 residue of Pik1p, which is required to bind Cdc4p and is required for *pik1*^{wt} ectopic lethality, is important for Pik1p function. We reported previously that the mutant allele *cdc4*^{G107S} also impaired the ability of Pik1p to interact with Cdc4p (Desautels *et al.*, 2001). It was then plausible that ectopic expression of *pik1*^{wt} may not be lethal in cells carrying the *cdc4*^{G107S} allele.

To test this hypothesis, ectopic expression of the wild-type and mutant alleles of *pik1* were then carried out in cells with genomic temperature-sensitive alleles of *cdc4*. Cells with the *cdc4*^{G107S} allele cultured at 30°C and carrying the pREP1 vector alone formed colonies in the presence and absence of thiamine. Western blot analysis was performed to determine if the *pik1* alleles accumulated in *cdc4*^{G107S} cells. As observed with the ectopic expression of the *pik1* alleles in wild-type cells, all the *pik1* alleles accumulated in *cdc4*^{G107S} cells (Figure 4.18). In the absence of thiamine, the *pik1*^{D709A, R838A} allele accumulated to lower levels than the other *pik1* alleles (Figure 4.18). This was observed consistently in the 3 western blots that were performed. As described in the western blots of the wild-type cells carrying the *pik1* alleles, the α -Pik1p antisera recognized a 93 kDa polypeptide in homogenates of cells carrying the vector alone cultured in the absence of thiamine (Figure 4.18). As discussed previously, this band is likely a cross-reactive protein in the cell homogenate. In cells in which the *pik1* alleles were ectopically expressed, a slightly larger band of 97 kDa was observed in addition to the ~93 kDa polypeptide (Figure 4.18). The larger band likely corresponds to the Pik1p protein. Additional bands in the range of 60 to 70 kDa were also observed and likely represent the break-down products of the ectopically expressed Pik1p protein, even though protease inhibitors were added (Figure 4.18). Overall, the wild-type and mutant Pik1p proteins accumulate in *cdc4*^{G107S} *S. pombe* cells. Furthermore, the levels of expression, with the exception of the *pik1*^{D709A, R838A} allele, appear to accumulate to levels similar to those in wild-type cells. However, the 93 kDa band appeared to accumulate to higher levels in *cdc4*^{G107S} cells, compared to wild-type

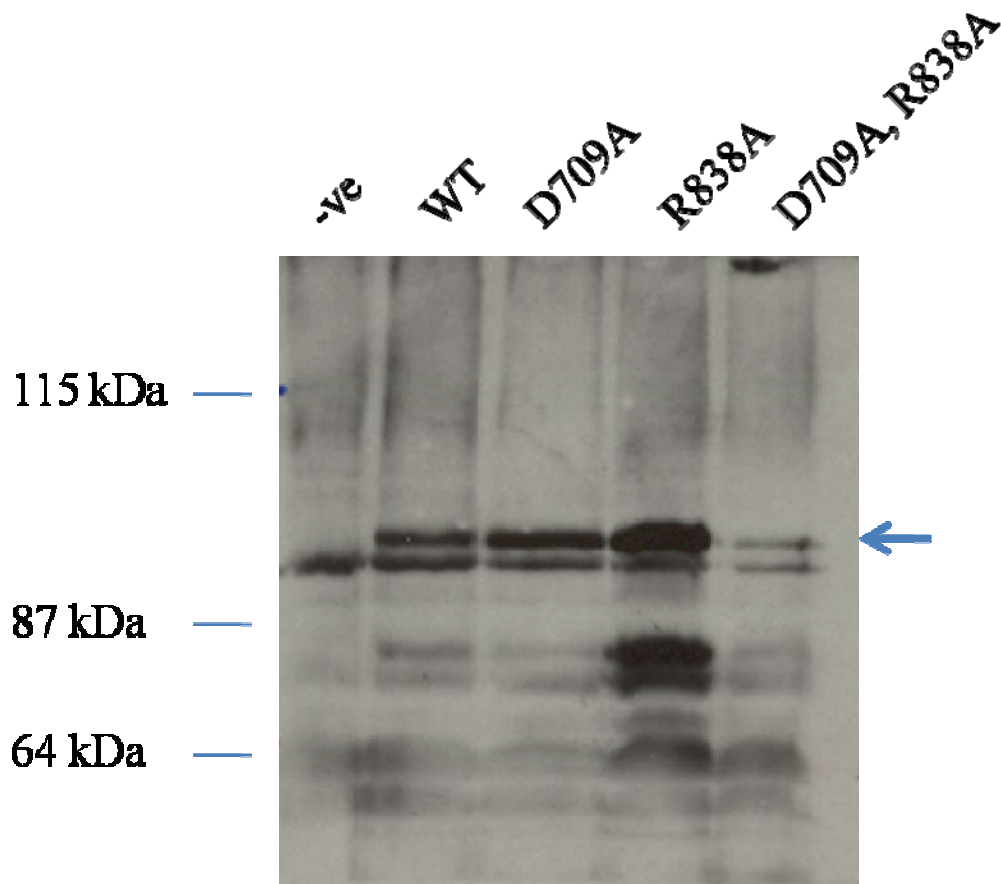


Figure 4.18: **Pik1p wild-type and mutant alleles accumulate in *S. pombe* *cdc4*^{G107S} cells.**

The *cdc4*^{G107S} cells were transformed with pREP1 plasmid with: no insert (-ve), wild-type *pik1* (WT), *pik1*^{D709A} (D709A), *pik1*^{R838A} (R838A) and *pik1*^{D709A, R838A} (D709A, R838A). The cells were cultured for 24 hours at 30°C in the absence of thiamine. Aliquots of cell homogenates (5 µg of protein) were subjected to denaturing polyacrylamide gel electrophoresis and immunoblot analysis with a rabbit anti-Pik1p primary antibody and a secondary HRP-conjugated antibody. The bars on the left indicate migration of the benchmark (Invitrogen) standard. Results are representative of 3 independent experiments. The arrow points to the ectopically expressed *pik1* protein product of 97 kDa.

cells, suggesting that a lack of Cdc4p interaction may cause the endogenous Pik1p protein to be protected from degradation, or be more stable.

No effect on colony formation was also observed in cells carrying the *pik1* wild-type allele on plates containing thiamine (Figure 4.19). The cells ectopically expressing the *pik1* wild-type sequence in *cdc4*^{G107S} cells grew similarly to *cdc4*^{G107S} cells carrying the vector alone or cultured under repressed conditions at 30°C (Figure 4.19). This is in marked contrast to the suppression of colony formation observed upon the ectopic expression of the *pik1*^{wt} allele in wild-type cells (Figure 4.12). Similar results were observed when the experiments were conducted at 19°C and 25°C (data not shown). Colony formation was observed at these temperatures to assess any temperature-sensitive effects that the ectopic expression of the *pik1* alleles might have.

Little to no effect on cell proliferation was also observed in liquid cultures of *cdc4*^{G107S} cells ectopically expressing the *pik1*^{wt} sequence compared to cells carrying the vector alone (Figure 4.20). This was an allele specific effect not observed with the ectopic expression of the *pik1* wild-type sequence in the other *cdc4* conditional strains (Figure 4.21). These *cdc4* conditional strains, like the *cdc4*^{G107S} allele, show cytokinesis defects when cultured at the restrictive temperature (35-37°C). All these alleles, except for *cdc4*^{G107S} interact with the C-terminal domain of Pik1p in a yeast two-hybrid assay. Thus, in the absence of the Pik1p-Cdc4p interaction, either by the *cdc4*^{G107S} mutation or the *pik1*^{R838A} mutation, *pik1*^{wt} ectopic lethality is not observed. It is interesting to note that there is an increased number of elongated cells in *cdc4*^{G107S} cells cultured in the presence and absence of thiamine carrying the vector alone at 25°C (Figure 4.22).

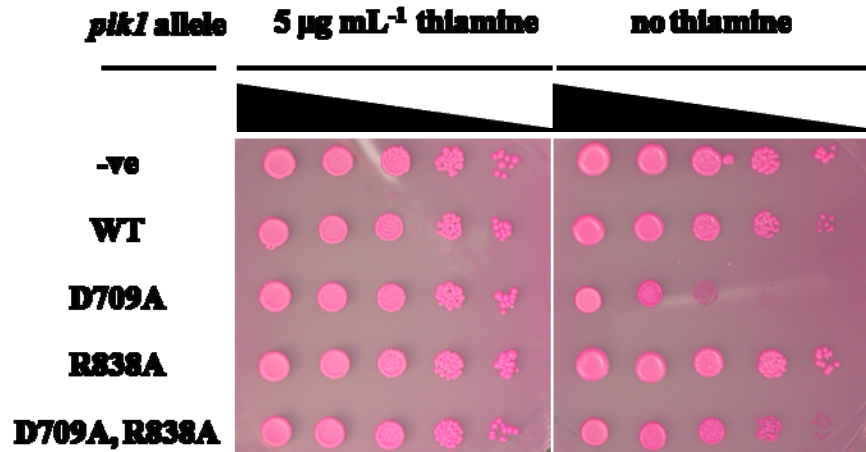


Figure 4.19: The *pik1* ectopic lethal phenotype is suppressed in *cdc4*^{G107S} cells.

Serial dilutions of *S. pombe cdc4*^{G107S} cells transformed with the pREP1 expression vector containing: no insert (-ve); *pik1* wild-type (WT); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A) were spotted onto EMM –leu plates that contained phloxin B. Aliquots (5 μL) of each dilution of 10^5 to 10^1 cells/mL were applied per spot (ramp). Culture medium was supplemented with thiamine or not as indicated and plates were incubated at 30°C for 6 days. Results shown are representative of 3 independent experiments.

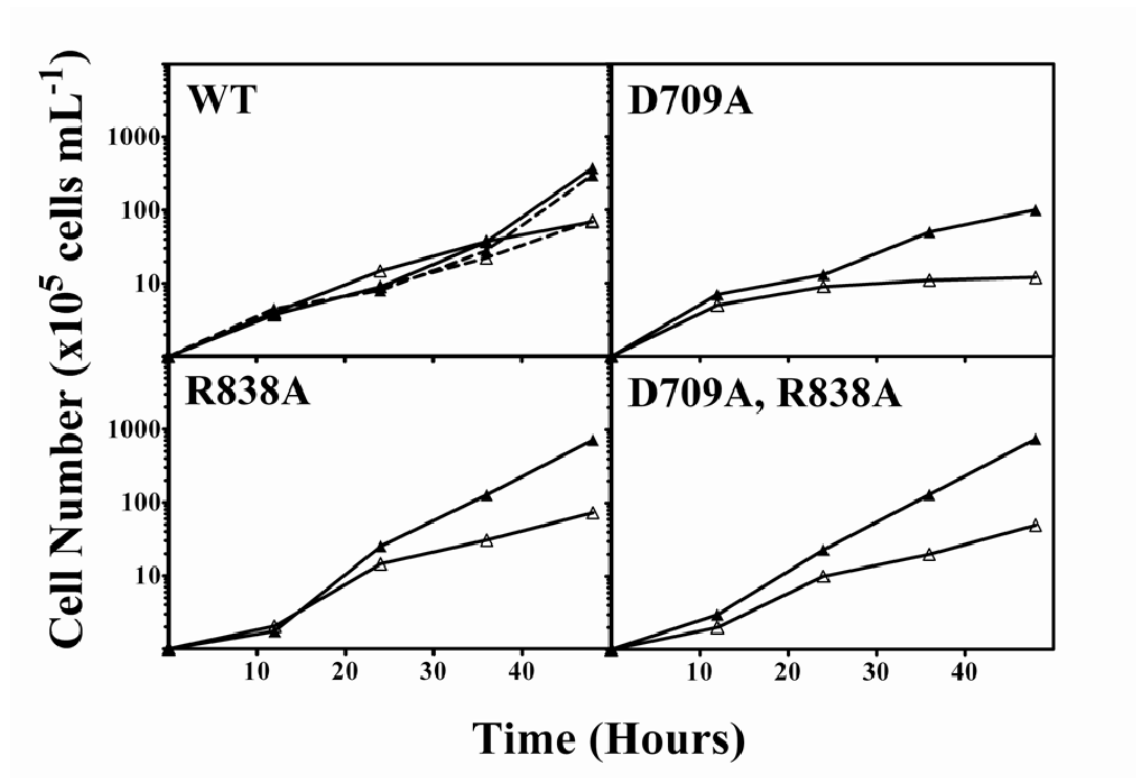
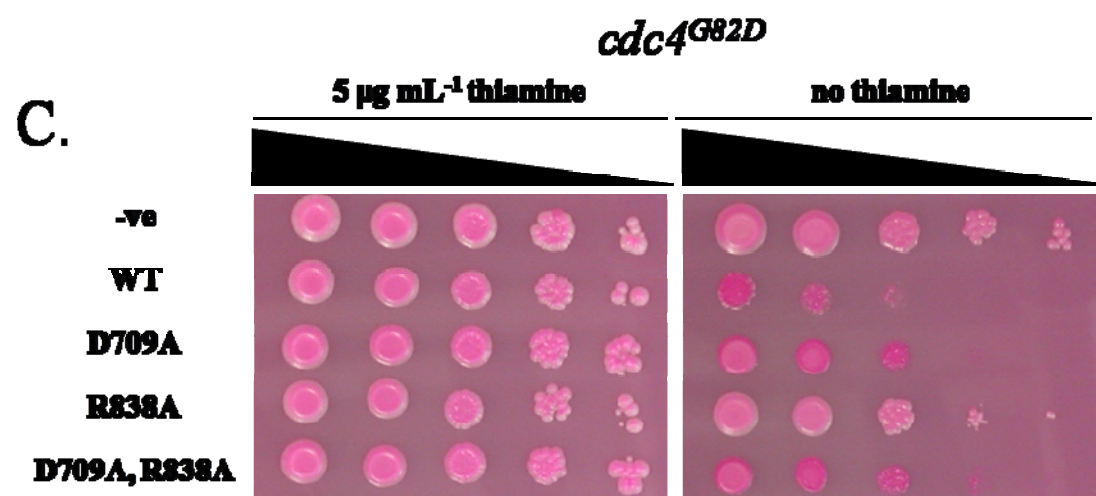
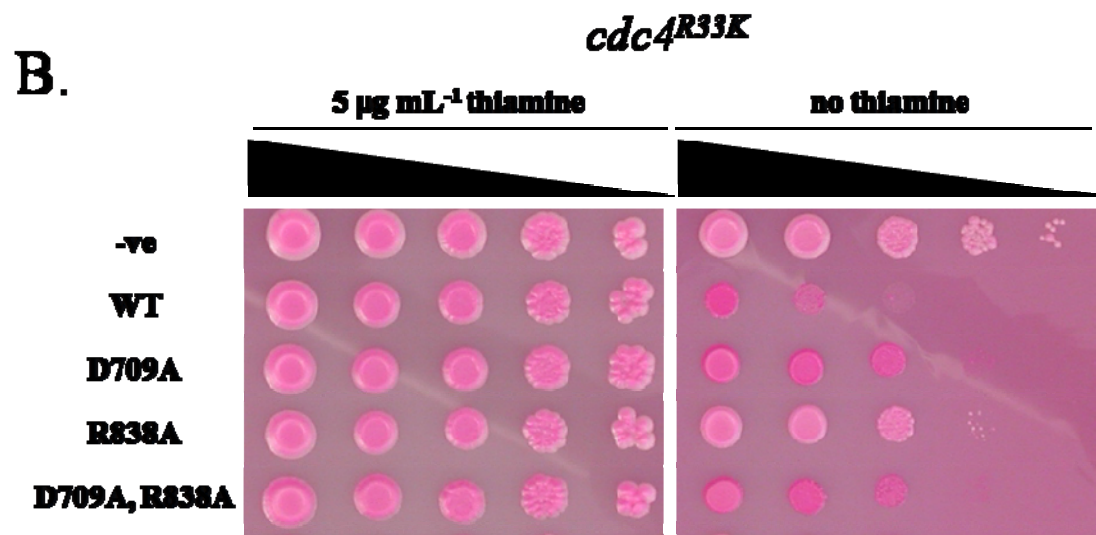
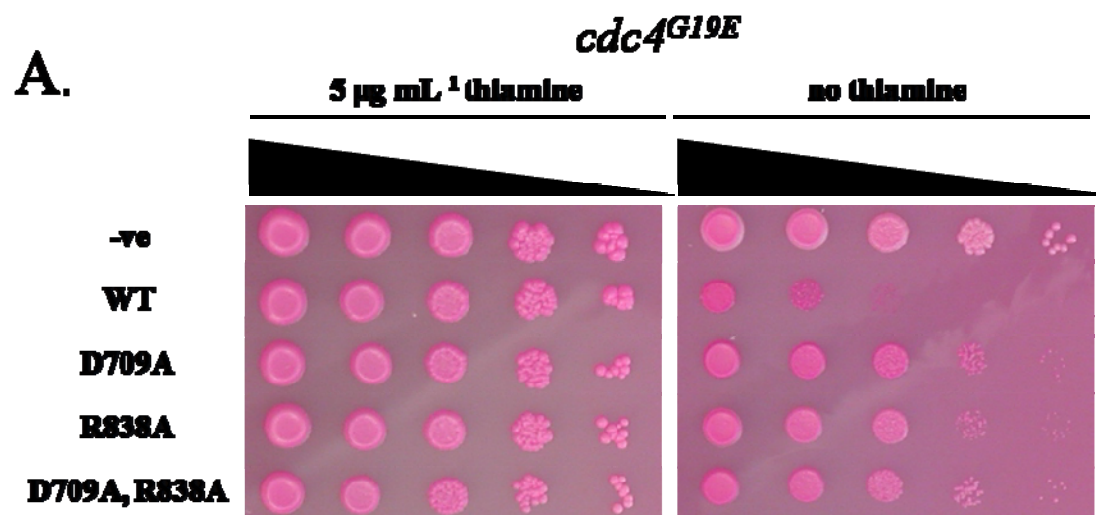


Figure 4.20: Cell proliferation after ectopic expression of *pik1* alleles in *cdc4*^{G107S} cells.

cdc4^{G107S} cells were transformed with the pREP1 vector containing: no insert (-ve); *pik1* wild-type (WT); *pik1*^{D709A} (D709A); *pik1*^{R838A} (R838A); or *pik1*^{D709A, R838A} (D709A, R838A) sequences. Cell cultures were started with a cell density of 1×10^5 cells/mL in EMM –Leu and incubated at 25°C in the presence (closed triangle) or absence (open triangle) of thiamine.



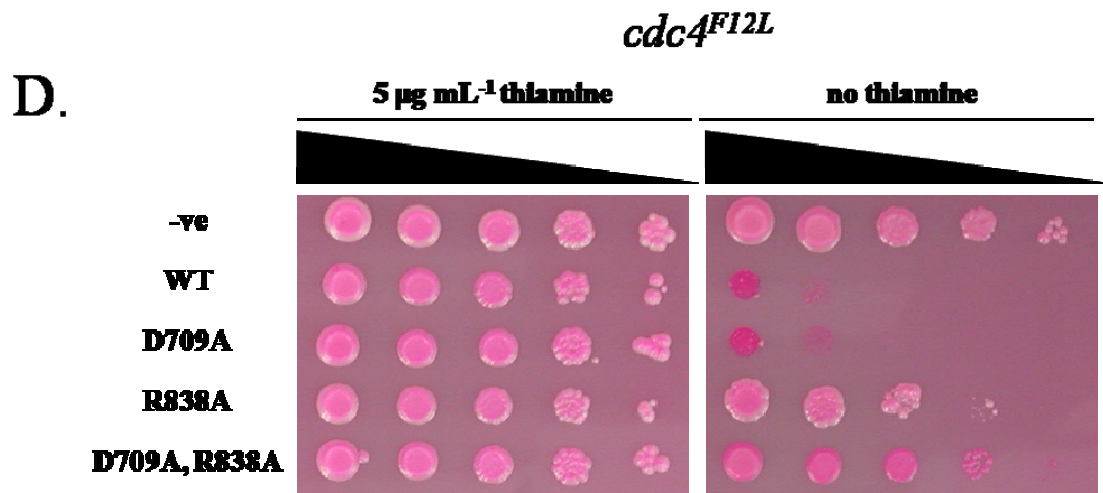


Figure 4.21: Colony formation after ectopic expression of *pik1* alleles in *cdc4* temperature-sensitive strains.

Serial dilutions of *S. pombe cdc4^{G19E}* cells (A), *cdc4^{R33K}* cells (B), *cdc4^{G82D}* cells (C) and *cdc4^{F12L}* cells (D) transformed with the pREP1 expression vector containing: no insert (-ve); *pik1* wild-type (WT); *pik1^{D709A}* (D709A); *pik1^{R838A}* (R838A); or *pik1^{D709A, R838A}* (D709A, R838A) were spotted onto EMM –Leu plates that contained phloxin B. Aliquots (5 μL) of each dilution of 10^5 to 10^1 cells/mL were applied per spot (ramp). Culture medium was supplemented with thiamine or not as indicated and plates were incubated at 30°C for 6 days. Results shown are representative of 3 independent experiments.

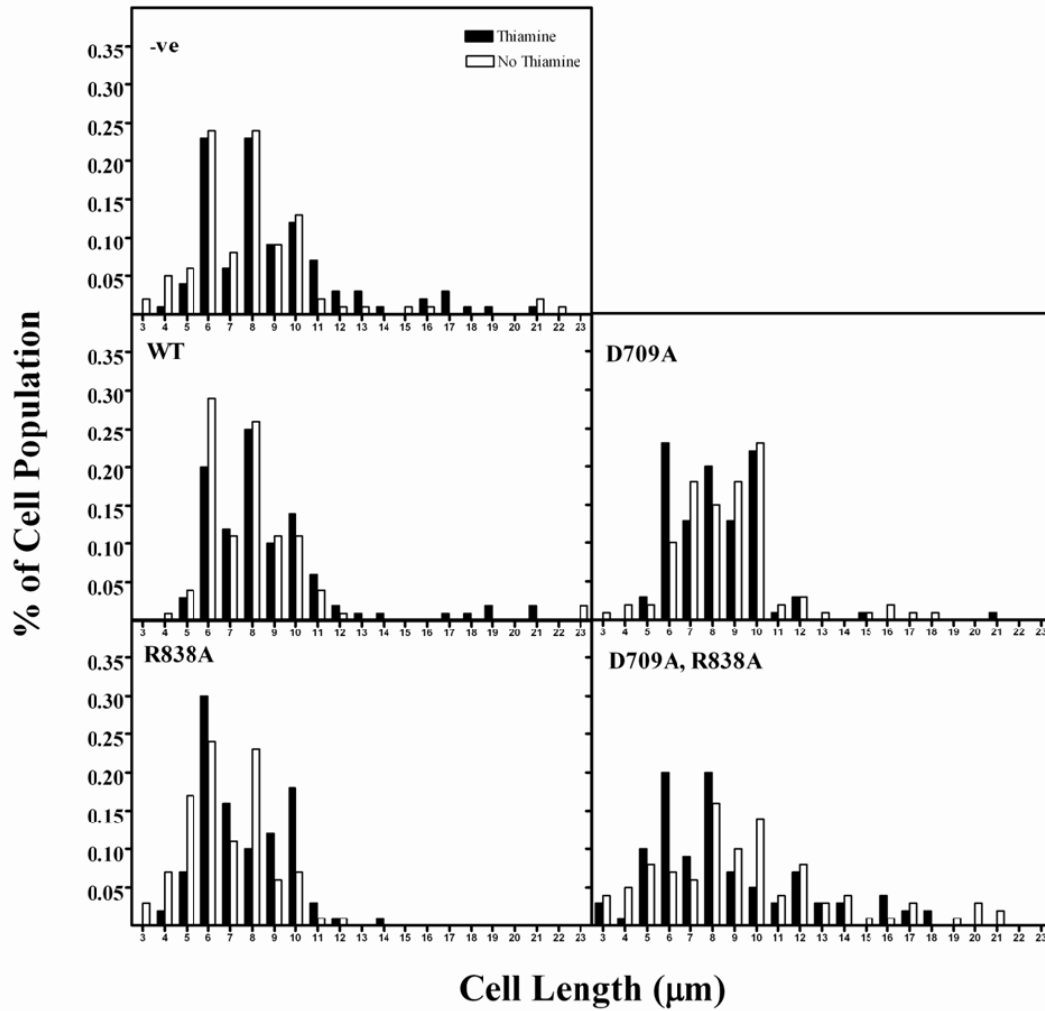


Figure 4.22: Histograms of cell length of *S. pombe cdc4^{G107S}* cells after ectopic expression of *pik1* alleles.

S. pombe cdc4^{G107S} cells were transformed with the expression vector containing: no insert (-ve), *pik1* wild-type (WT); *pik1^{D709A}* (D709A); *pik1^{R838A}* (R838A); or *pik1^{D709A, R838A}* (D709A, R838A). Cell length was measured from a population of cells grown for 24 hours at 25°C in the presence (black bars) or absence (white bars) of thiamine. Cell length was measured from photographs of cells stained with CF+DAPI relative to a micrometer bar. About 100 cells were measured from a single culture for each allele in repressed and derepressed conditions.

Thus, even at the permissive temperature, a small proportion of the population has cytokinesis defects.

Upon the ectopic expression of the *pikI*^{wt} allele in *cdc4*^{G107S} cells, there were no significant effects on cell morphology, cell length, septum formation, actin patch formation or contractile ring formation (Table 4.4). This is in contrast to *cdc4*⁺ cells ectopically expressing the *pikI* wild-type allele which demonstrated a dramatic decrease in the formation of actin and myosin rings.

4.8.9. The ectopic expression of *pikI*^{D709A} impairs cell proliferation in *cdc4*^{G107S} cells

Ectopic expression of the *pikI*^{D709A} allele in *cdc4*^{G107S} cells had a marked effect on colony formation (Figure 4.19). This is in contrast to the ectopic expression of the same allele in *cdc4*⁺ cells which only mildly affected colony formation. In liquid culture, ectopic expression of the *pikI*^{D709A} allele arrested cell proliferation after 24 hours at 25°C (Figure 4.20).

The cells had a similar appearance under bright field microscopy compared to cells carrying the vector alone under derepressed and repressed conditions (data not shown). The proportion of the cell population with F-actin rings and calcofluor staining in the medial region of the cell were also similar to cells carrying the vector alone or cultured in repressed conditions (Table 4.4). The mean length of cells ectopically expressing the *pikI*^{D709A} allele increased only slightly from $8.1 \pm 0.2 \mu\text{m}$ in repressed conditions to $8.6 \pm 0.3 \mu\text{m}$ in derepressed conditions (Table 4.5). Also, there was no effect on cell length distributions upon the ectopic expression of the *pikI*^{D709A} allele (Figure 4.22). Thus, ectopic expression of the *pikI*^{D709A} allele in *cdc4*^{G107S} cells

Table 4.4.

Effects of ectopic expression of *pik1* alleles on characteristics of cytokinesis in *S. pombe* cells with the ts-allele *cdc4*^{G107S}

	+ thiamine			no thiamine		
	CR ^{Actin}	Septa (frequency, %)	BiN	CR ^{Actin}	Septa (frequency, %)	BiN
-	11	18	20	11	18	20
WT	10	17	23	10	16	18
D709A	9	17	23	15	20	29
R838A	9	15	19	9	25	24
D709A, R838A	10	15	23	8	26	35

Cell cultures were at 25°C for 24 hours with or without thiamine. Cells were evaluated for the presence of contractile ring (CR, visualized with FITC-phalloidin for F-actin), septa (visualized with calcofluor-white) and two nucleus (BiN with DAPI). Results are expressed as % of the cell population examined with n=100 cells.

Table 4.5.Effects of ectopic expression of *pik1* alleles on mean cell length (μm) of *cdc4*^{G107S} cells

Allele	+ thiamine	no thiamine
-	8.8 \pm 0.3	7.8 \pm 0.3
<i>pik1</i>	8.6 \pm 0.3	7.8 \pm 0.3
<i>pik1</i> ^{D709A}	8.1 \pm 0.2	8.6 \pm 0.3
<i>pik1</i> ^{R838A}	7.5 \pm 0.2	6.6 \pm 0.2
<i>pik1</i> ^{D709A,R838A}	8.7 \pm 0.4	9.4 \pm 0.4

Cell length was measured from a population of cells grown for 24 hours at 25°C in the presence or absence of thiamine. Cell length was measured from photographs of cells stained with CF+DAPI relative to a micrometer bar. The length of 100 cells was measured from a single culture for each allele.

resulted in increased $\text{Pik1p}^{\text{D709A}}$ protein accumulation and suppressed cell proliferation, but without any apparent effect on several indicators of cytokinesis.

4.8.10. The ectopic expression of $\text{pik1}^{\text{R838A}}$ in $\text{cdc4}^{\text{G107S}}$ cells

Ectopic expression of the $\text{pik1}^{\text{R838A}}$ allele in $\text{cdc4}^{\text{G107S}}$ cells had little to no effect on colony formation (Figure 4.19). Cells ectopically expressing the $\text{pik1}^{\text{R838A}}$ allele formed colonies comparable to cells carrying the vector alone, or the same cells grown in repressed conditions. This was comparable to the ectopic expression of the same allele in cdc4^+ cells which only mildly affected colony formation. In liquid culture, cells ectopically expressing the $\text{pik1}^{\text{R838A}}$ allele continued to proliferate after 24 hours, albeit more slowly than cells cultured in the presence of thiamine (Figure 4.20).

The cells had a similar appearance under bright field microscopy compared to cells carrying the vector alone under derepressed and repressed conditions (data not shown). The proportion of the cell population with F-actin rings and CF staining in the medial region was also similar to cells carrying the vector alone or cultured in repressed conditions (Table 4.4).

The mean length of cells ectopically expressing the $\text{pik1}^{\text{R838A}}$ allele was significantly decreased and this effect was observed even in repressed conditions (Figure 4.22, Table 4.5). Indeed, a small proportion of $\text{cdc4}^{\text{G107S}}$ cells carrying the vector alone were elongated (within the range of 15-22 μm) (Figure 4.22). No elongated cells were observed in cells carrying the $\text{pik1}^{\text{R838A}}$ allele cultured in the presence or absence of thiamine (Figure 4.22). This was comparable to the effect

observed with the expression of the same allele in wild-type cells where a high proportion of the cell population were shorter in length (Figure 4.15).

4.8.11. The ectopic expression of the *pikI*^{D709A, R838A} allele in *cdc4*^{G107S} cells

Ectopic expression of the *pikI*^{D709A, R838A} allele in *cdc4*^{G107S} cells had a slight effect on colony formation (Figure 4.19). In liquid culture, cells ectopically expressing the *pikI*^{D709A, R838A} allele continued to proliferate after 24 hours. However, the cells ectopically expressing the *pikI*^{D709A, R838A} allele proliferated more slowly than cells cultured in the presence of thiamine (Figure 4.20).

The cells had a similar appearance under bright field microscopy compared to cells carrying the vector alone cultured under derepressed and repressed conditions (data not shown). The proportion of the cell population with F-actin rings and calcofluor staining in the medial region were also similar to cells carrying the vector alone or cultured in repressed conditions (Table 4.4). The mean length of cells ectopically expressing the *pikI*^{D709A, R838A} allele increased slightly compared to cells carrying the vector alone or cultured in repressed conditions, similar to *cdc4*^{G107S} cells ectopically expressing the *pikI*^{D709A} allele (Table 4.5). The cells however, proliferated at a higher rate than cells ectopically expressing the *pikI*^{D709A} single mutant.

In summary, removal of the Pik1p-Cdc4p interaction, either through the *cdc4*^{G107S} allele, or the ectopic expression of the *pikI*^{R838A} allele, removes *pikI*^{wt} ectopic lethality. Ectopic expression of the *pikI*^{D709A} allele in *cdc4*^{G107S} cells is lethal, by an as of yet unknown mechanism. The ectopic expression of the *pikI*^{R838A} allele in *cdc4*^{G107S} cells results in a slower rate of cell proliferation and cell length shortening.

4.9. Does Cdc4p regulate Pik1p lipid kinase activity?

4.9.1. Upon the ectopic expression of the *pik1^{wt}* and *pik1^{R838A}* alleles, an increase in lipid kinase activity is not observed in *cdc4^{G107S}* cells

Ectopic expression of *pik1^{wt}* in *cdc4^{G107S}* cells has a limited effect on cell proliferation. It was then of interest to test if *cdc4^{G107S}* cells with ectopically expressed *pik1^{wt}* had altered lipid kinase activity. Here, 0.8 µg of *cdc4^{G107S}* cell homogenates carrying expressed *pik1^{wt}* and *pik1^{R838A}* protein were pulsed with ³²P-γ-ATP and the radiolabelled phospholipid product extracted and separated by TLC. The thiamine-responsive radiolabeled spot corresponding to monophosphorylated PtdIns was then scraped and measured by liquid scintillation counting. The apparent endogenous incorporation of ³²P into monophosphorylated PtdIns by *cdc4^{G107S}* cell homogenates carrying the vector alone was lower than that observed in wild-type cell homogenates (1349±171 DPM from 2887±810 DPM in wild-type cells) (Figure 4.23). Upon the ectopic expression of the *pik1^{wt}* allele in wild-type cells, a 3-fold increase in the incorporation of ³²P into monophosphorylated PtdIns was observed as described previously. This was not seen with the ectopic expression of *pik1^{wt}* in *cdc4^{G107S}* cells (1584±360 DPM compared to wild-type cells where *pik1* lipid kinase activity reached 8259±229 DPM) (Figure 4.23). Likewise, when the *pik1^{R838A}* allele was ectopically expressed in *cdc4^{G107S}* cells, the incorporation of ³²P into monophosphorylated PtdIns failed to increase to levels observed with the ectopic expression of the same sequence in wild-type cells (3456±2253 DPM compared to the lipid kinase activity of *pik1^{R838A}* in wild-type cells, 11780±5769 DPM) (Figure 4.23). The incorporation of ³²P into monophosphorylated PtdIns of *cdc4^{G107S}* cell homogenates carrying the expressed

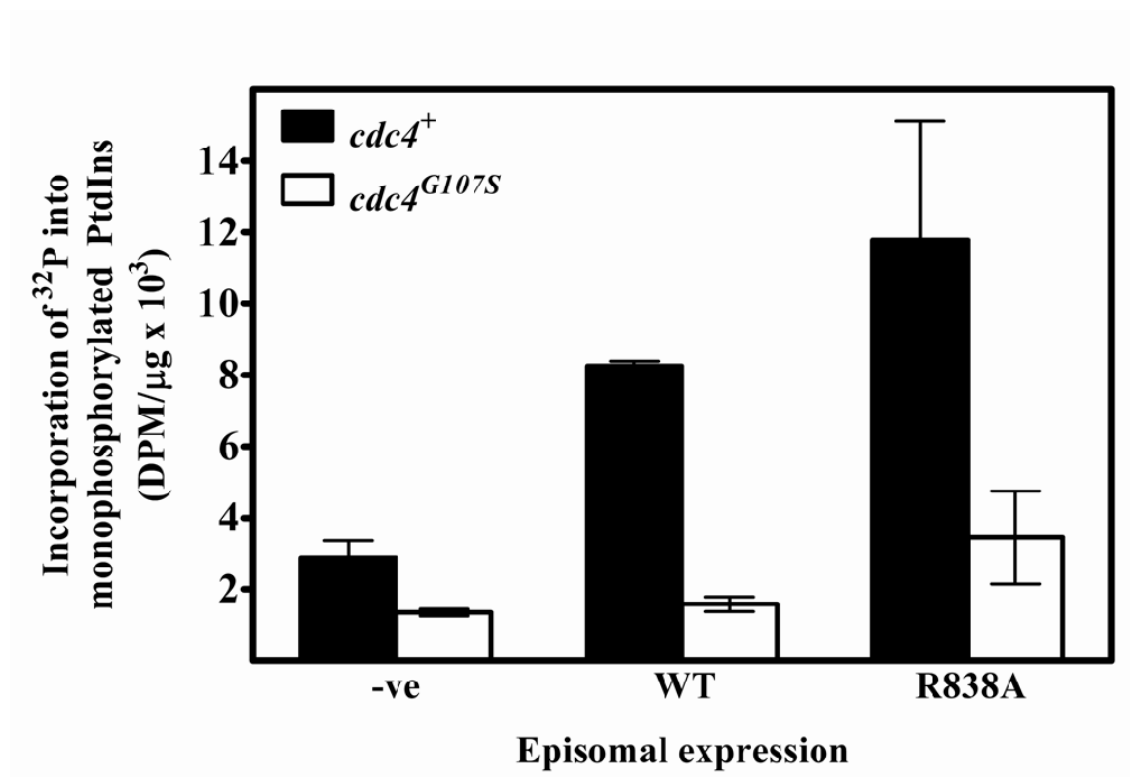


Figure 4.23: *In vitro* lipid kinase activity in homogenates of wild-type and *cdc4*^{G107S} cells after ectopic expression of *pik1* or *pik1*^{R838A}.

Homogenates were from wild-type (black bars) or *cdc4*^{G107S} cells (white bars) carrying the pREP1 expression vector with no insert (-ve), *pik1*^{wt} expression vector (WT) or *pik1*^{R838A} expression vector (R838A). The cultures were grown for 24 hours at 25°C in medium that lacked thiamine. ³²P incorporation into monophosphorylated PtdIns was reported as DPM/μg of total protein. Results are shown as the mean ±S.E. for 3 independent experiments.

pik1^{D709A} protein was not measured as this allele was shown to not have lipid kinase activity. The reduced incorporation of ^{32}P into monophosphorylated PtdIns upon the ectopic expression of the *pik1^{wt}* and *pik1^{R838A}* allele in *cdc4^{G107S}* cells was an allele specific effect. The incorporation of ^{32}P into monophosphorylated PtdIns was mildly affected upon the ectopic expression of the *pik1^{wt}* and *pik1^{R838A}* alleles in the other *cdc4* conditional strains (Figure 4.24). Homogenates from five temperature-sensitive strains of *cdc4* were tested for the increased incorporation of ^{32}P into monophosphorylated PtdIns upon the ectopic expression of *pik1^{wt}* and *pik1^{R838A}*. Of the four other *cdc4* temperature-sensitive alleles, ectopic expression of the *pik1^{wt}* and *pik1^{R838A}* sequences caused variable levels of the incorporation of ^{32}P into monophosphorylated PtdIns (Figure 4.24). The exception to this was *cdc4^{G19E}*, which displayed significantly lower endogenous incorporation of ^{32}P into monophosphorylated PtdIns and reduced incorporation of ^{32}P into monophosphorylated PtdIns of the *pik1^{R838A}* allele (Figure 4.24). The cells were cultured in identical conditions to the *cdc4^{G107S}* mutant.

Lipid kinase assays with purified Cdc4p protein were also performed. Various concentrations of Cdc4p purified protein (0.1-10 mM Cdc4p) were added to 0.4 μg of cell homogenates carrying expressed Pik1p protein. No effect on the incorporation of ^{32}P into monophosphorylated PtdIns in these cell homogenates were observed (data not shown). Thus, *cdc4^{G107S}* cells, known to have the most penetrant effect on cytokinesis, are the least responsive to increased lipid kinase activity upon the ectopic expression of the *pik1^{wt}* and *pik1^{R838A}* alleles.

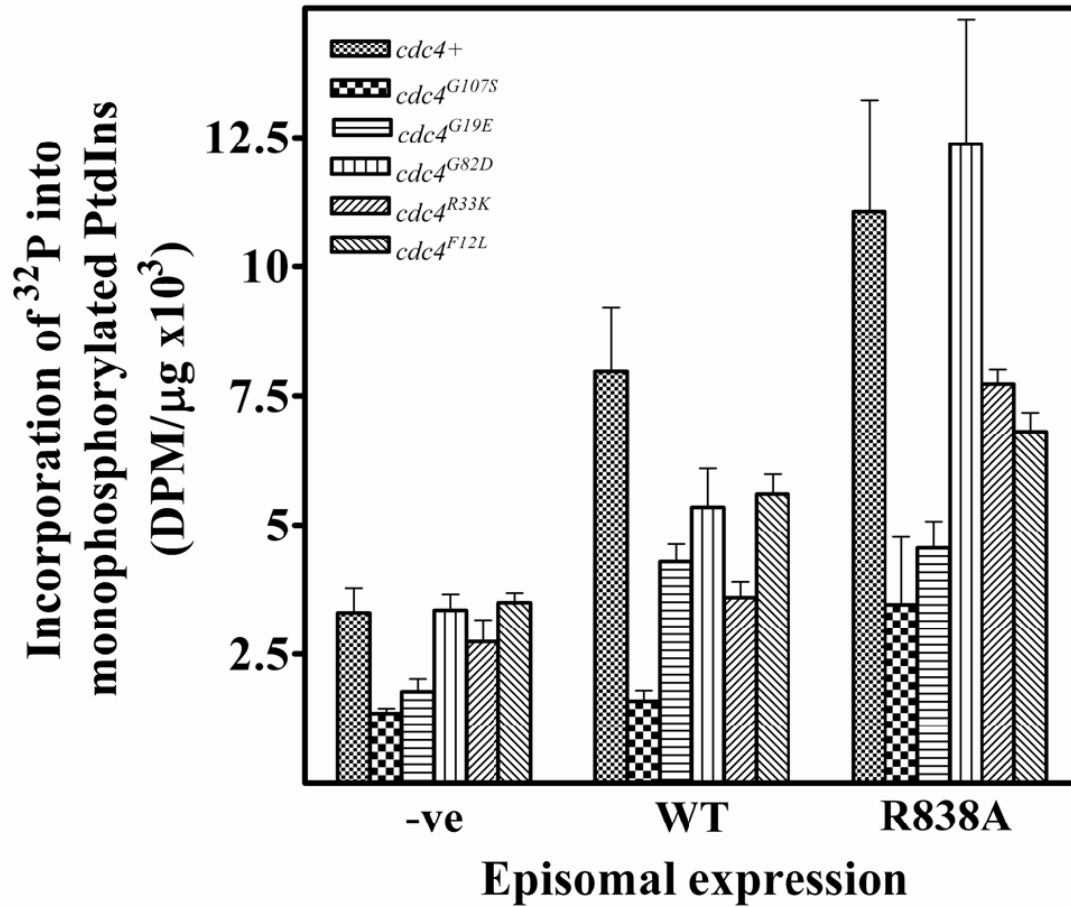


Figure 4.24: *In vitro* lipid kinase activity in homogenates of wild-type and *cdc4* conditionally lethal strains after ectopic expression of *pik1* or *pik1*^{R838A}.

Homogenates were from wild-type cells or *cdc4*^{G107S}, *cdc4*^{G19E}, *cdc4*^{G82D}, *cdc4*^{R33K} and *cdc4*^{F12L} cells carrying the pREP1 expression vector with no insert (-ve), *pik1*^{wt} allele (WT) or *pik1*^{R838A} allele (R838A). The cultures were grown for 24 hours at 25°C in medium that lacked thiamine. ^{32}P incorporation into monophosphorylated PtdIns was reported as DPM/ μg of total protein. Results are shown as the mean \pm S.E. for 3 independent experiments.

Chapter 5: Discussion

5.1. Pik1p is a PtdIns 4-kinase

S. pombe Pik1p was identified as a putative type III β PtdIns 4-kinase based on sequence comparisons of the primary structure to those of enzymes experimentally shown to be type III β PtdIns 4-kinases. The human and bovine type III β PtdIns 4-kinase homologues were characterized enzymatically by expressing recombinant GST-tagged versions of each protein in *E. coli* and analyzing the lipid kinase activity of the purified protein (Meyers and Cantley, 1997; Zhao *et al.*, 2000). The homologue in *Arabidopsis thaliana* was also characterized enzymatically as being a type III β PtdIns 4-kinase by expressing and purifying the protein from baculovirus-infected insect cells (*Spodoptera frugiperda*) (Xue *et al.*, 1999). Finally, the *S. cerevisiae* PtdIns4KIII β homologue, Pik1p, was established as a PtdIns 4-kinase through activity assays of crude extracts from cells in which the gene was overexpressed (Garcia-Bustos *et al.*, 1994) and activity assays of the Pik1p protein after immunoprecipitation (Walch-Solimena and Novick, 1999). Experimental evidence presented in this thesis suggests that *S. pombe* Pik1p is a type III β PtdIns 4-kinase, although, it does not exclude the possibility

that Pik1p is a PtdIns 3-kinase. The latter possibility is not well supported by sequence comparisons (Figures 4.1 and 4.2).

The lipid kinase activity assay developed for this study measured the incorporation of ^{32}P into endogenous monophosphorylated PtdIns in crude cell homogenates after pulse labeling with ^{32}P - γ -ATP. This assay measured the incorporation of ^{32}P into monophosphorylated PtdIns since the thiamine-responsive labeled lipid product that was isolated by thin layer chromatography, co-migrated with the PtdIns3P and PtdIns4P lipid standards.

Since the lipid fraction that was analyzed for the incorporation of ^{32}P corresponded to both PtdIns3P and PtdIns4P monophosphorylated PtdIns, and since the labeling experiments were conducted with crude homogenates of cells, which contains many PtdIns metabolizing enzymes, the activity measured may have represented the combined activities of the three PtdIns 4-kinases, three PtdIns 3-kinases, two 3-phosphatases, and two 5-phosphatases in *S. pombe*.

There is an additional monophosphorylated PtdIns, PtdIns5P. This phosphoinositide also migrates with PtdIns3P and PtdIns4P using the developing system used for this study (1-propanol: 2 M acetic acid) and is difficult to separate from PtdIns4P (Pendaries *et al.*, 2005). The presence of this phosphoinositide has not been observed in *S. cerevisiae* (Gary *et al.*, 1998; Sbrissa *et al.*, 1999). In mammalian cells, the presence of two enzymes have been shown to lead to the production of PtdIns5P. They are the PIKfyve enzyme, of which there is a homologue in *S. cerevisiae*, Fab1p, and the myotubularin family of 3-phosphatases, which produce PtdIns5P through the dephosphorylation of PtdIns(3,5)P₂, of which there is also a homologue in *S. cerevisiae*,

Ymr1p (Shisheva, 2001; Walker *et al.*, 2001; Sbrissa *et al.*, 2002; Tronchere *et al.*, 2003, 2004). These two proteins are also present in *S. pombe*. Thus, considering the presence of the yeast homologues of these enzymes in the yeast genome, and considering that *S. pombe* genes are more closely related to their mammalian homologues, PtdIns5P may be present in *S. pombe*. However, even in mammalian cells, PtdIns5P is present in very low quantities (1-5% of total PtdInsP) (Rameh *et al.*, 1997a and b; Tolias *et al.*, 1998; Pendaries *et al.*, 2005). If PtdIns5P is present in *S. pombe*, then we can also not exclude that Pik1p is a PtdIns 5-kinase, although this is not supported by the primary sequence structure of *S. pombe* Pik1p (Figure 4.2).

Ectopic expression of the *pik1^{wt}* allele caused an increase in the incorporation of ³²P into monophosphorylated PtdIns, approximately 3-fold greater than for cells carrying the vector alone. Mutation of a residue in the catalytic region of Pik1p thought to be important for ATP binding and phosphotransfer (D709 to A) abrogated the increase in the production of labeled monophosphorylated PtdIns. In fact, ectopic expression of the *pik1^{D709A}* sequence acted to decrease the incorporation of ³²P into monophosphorylated PtdIns. Ectopic expression of a kinase-dead allele may act to sequester endogenous PtdIns leaving less available for phosphorylation by the other PtdIns 4-kinases, PtdIns 3-kinases and possibly the PtdIns 5-kinase in the cell homogenate. The binding constants for PtdIns of these mutant forms of PtdIns kinases has not been established. However, this particular mutation, since it affects ATP binding and phosphotransfer, is not expected to affect its interaction with PtdIns. These results support the conclusion that Pik1p is PtdIns 4-kinase because ectopic expression of the *pik1^{wt}* sequence increased the lipid kinase activity in crude homogenates of cells,

the level of the monophosphorylated labeled lipid product was thiamine-responsive and it co-migrated with PtdIns4P, and the ectopic expression of an allele that would be expected to be PtdIns 4-kinase-null failed to produce activity and in fact, it suppressed the apparent endogenous activity.

The assays were performed using crude homogenates of cells, which also contained the other PtdIns metabolizing enzymes that are expressed in the vegetative life cycle during log phase growth as described above. Also, the radiolabeled lipid fraction recovered after thin layer chromatography was thiamine-responsive, but may have been a mixture of labeled PtdIns4P, labeled PtdIns3P and perhaps labeled PtdIns5P. Thus, it cannot be concluded whether the observed increase in monophosphorylated PtdIns was due to the direct consequence of *pik1^{wt}* ectopic expression or the indirect consequence of *pik1^{wt}* ectopic expression through the induction of other PtdIns metabolizing enzymes in the cell homogenate. To circumvent these problems, an alternative developing system can be used to resolve PtdIns3P from PtdIns4P. This can be performed by introducing boric acid into the thin layer chromatography developing solution (Garcia-Bustos *et al.*, 1994). Boric acid forms a borate complex with the 2,3 *cis*-diols in PtdIns4P which allows PtdIns3P to migrate away from the borate-PtdIns4P complex (Mazzeo and Krull, 1989; Walsh *et al.*, 1999). Also, the indirect effects of this assay could be ruled out if the *pik1^{wt}* enzyme were purified through immunoprecipitation and if an exogenous PtdIns substrate was added. In this assay, yeast PtdIns should be used as a substrate since vertebrate PtdIns is polyunsaturated. This may affect the recognition of PtdIns by Pik1p. Alternatively,

phosphorylation of PtdIns could be monitored through the introduction of labeled PtdIns or phosphorus-32 into the growth medium.

An independent line of experimental evidence strongly supports the identity of Pik1p as a PtdIns 4-kinase. The heterologous expression of *S. pombe pik1^{wt}* was able to complement the lethal phenotype of the *S. cerevisiae PIK1* temperature-sensitive strain *pik1-101* at the non-permissive temperature (Park, 2007). The *pik1-101* allele was shown to have greatly reduced lipid kinase activity (~95% lower than the wild-type enzyme) at 37°C (Walch-Solimena and Novick, 1999). Furthermore, the *S. pombe* lipid kinase null sequence (*pik1^{D709A}*) was unable to restore growth at 37°C (Park, 2007). These results indicate that the *S. pombe* cDNA is likely providing the *pik1-101* mutant with the lipid kinase activity required for colony formation at the restrictive temperature, and that this lipid kinase activity is essential for *PIK1* function. Indeed, *S. cerevisiae* Pik1p lipid kinase activity is required for PtdIns4P production at both the Golgi and nucleus and these activities are essential for viability (Strahl *et al.*, 2005). Consistent with this result, an additional *S. pombe pik1* allele, *pik1^{R838A}*, which was shown to have lipid kinase activity, but no Cdc4p-binding activity, was able to complement the *pik1-101* temperature-sensitive phenotype. I contributed to this study by providing the *pik1* cDNA and *pik1* mutant alleles. I also characterized the lipid kinase activities and Cdc4p-binding activities of the *pik1* alleles that I generated.

In summary, based on the experimental evidence presented here and on that presented by Park (2007) to which I contributed, it can be concluded that Pik1p is a lipid kinase, specifically a PtdIns 4-kinase, as has been suggested on the basis of primary structure comparisons.

5.2. Pik1p is localized to the Golgi and medial region in *S. pombe* cells

S. pombe Pik1p localizes to the Golgi and medial region during the late stages of cytokinesis (Park, 2007). The Golgi localization of Pik1p was confirmed by a global localization study performed by Matsuyama and colleagues (2006). Pik1p in *S. cerevisiae* also localizes to the nucleus (Walch-Solimena and Novick, 1999; Strahl *et al.*, 2005). The localization of *S. pombe* Pik1p to the nucleus was not observed by either Park (2007) or Matsuyama (2006). It is interesting to note that *S. cerevisiae* Pik1p contains a nuclear localization signal (K491KGK) in that it is lysine rich with proline residues and a casein kinase II site in close proximity (Garcia-Bustos *et al.*, 1994). This area appears to be weakly conserved in *S. pombe* Pik1p in that it is fairly rich in lysine residues, but with no proline residues in close proximity. Thus, it is possible that *S. pombe* Pik1p localizes to the nucleus, but in levels too low to permit detection through N-terminal tagging or indirect immunolocalization. Since Pik1p lipid kinase activity in the nucleus is essential for *S. cerevisiae* cell viability, and since *S. pombe pik1* was able to complement the *S. cerevisiae pik1-101* strain, it is likely that the *S. pombe pik1* sequence is being targeted to the nucleus as well. Another possibility is that the *S. cerevisiae pik1-101* allele could still be localizing to the nucleus and it contains sufficient activity to allow cell survival in the presence of the *S. pombe pik1* allele. The role of *S. cerevisiae* Pik1p in the nucleus is currently unknown (Strahl *et al.*, 2005).

Park showed that an N-terminal tandemly tagged eGFP Pik1p protein localized to the medial region of the cell late in cytokinesis in 30% of cells synchronized by

cdc25-22 block and release. The cell division index peaked at approximately 80%. The low frequency of Pik1p fluorescence at the medial region may reflect that Pik1p levels are near the level of detection for this assay. It may also indicate poor synchrony of Pik1p localization to the medial region by the block and release method using *cdc25-22* cells. It is unlikely that this medial localization is associated with the contractile ring since Pik1p localization to the medial region occurs at about the time of septum formation, but clearly after contractile ring formation. It is currently unknown to which structures at the medial region Pik1p associates with. Pik1p might be associated with the Golgi, other vesicles, the plasma membrane or the septum. Pik1p may be associated with the Golgi at the division site. It is still unknown if the Golgi in *S. pombe* partitions during the cell cycle, and if this partitioned Golgi is targeted to the division site during cytokinesis, like in *S. cerevisiae* (Rossanese *et al.*, 2001). It is possible that Pik1p is localized to Golgi that are transiently translocated to the medial region during cell division. Alternatively, Golgi derived vesicles may move to the medial region, or Pik1p could be shuttled to the medial cortex or septum.

One metabolite in particular, PtdIns(4,5)P₂, is found at the medial region of dividing cells (Janetopoulos *et al.*, 2005; Field *et al.*, 2005; Zhang *et al.*, 2000). Two enzymes which contribute to the PtdIns pool at the medial region in *S. pombe* cells are Ptn1p, a lipid phosphatase that dephosphorylates PtdIns(3,4,5)P₃ and Its3p, a PtdIns4P 5-kinase. Ptn1p localizes to the septum and Its3p is also found at this site, presumably involved in the formation of PtdIns(4,5)P₂ for cell division (Zhang *et al.*, 2000; Mitra *et al.*, 2004; Janetopoulos *et al.*, 2005). Indeed, PtdIns(4,5)P₂ production is important for cytokinesis since the hydrolysis of PtdIns(4,5)P₂ or interference of PtdIns(4,5)P₂ lipid–

protein interactions in various cell types causes cytokinesis defects (Janetopoulos and Devreotes, 2006).

In *cdc25-22* cells, PtdIns(4,5)P₂ localizes to the medial region at about the same time as contractile ring formation (Desautels *et al.*, unpublished observation). Since PtdIns(4,5)P₂ localizes to the medial region early in cytokinesis, other PtdIns4P enzymes may be required at earlier steps since Pik1p localizes to the medial region at about the same time as septum formation. Or perhaps, PtdIns4P is generated at the Golgi and trafficked to the medial region for use by other phosphoinositide metabolizing enzymes. This is supported by the observation that Pik1p-dependent PtdIns4P synthesis at the Golgi has been shown to be important for membrane trafficking (Nguyen *et al.*, 2005; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). Also, membrane trafficking is likely to be significant for membrane expansion and septation at the medial region during cytokinesis in *S. pombe*. For example, for the targeting of glucanases and enzymes required for septum synthesis to the medial region, to digest the septum and for the generation of septum, respectively.

5.3. Pik1p PtdIns 4-kinase activity is essential for *S. pombe* cell division

Pik1p PtdIns 4-kinase activity is essential for *S. pombe* cell viability as spores carrying the chromosomal *pik1*^{D709A} allele germinated, but failed to proliferate. Furthermore, the haploid cells which developed from the spores appeared to be elongated at 30°C suggesting a cytokinesis defect. Thus, the discrete production and localization of PtdIns metabolites at the Golgi and medial region is important for cell growth and division. Cells with the double-mutant form of *pik1* (*pik1*^{D709A,R838A})

integrated at the *pik1* genomic locus, which was shown to lack PtdIns 4-kinase activity, failed to proliferate. Most of the spores failed to germinate, although, two spores formed microcolonies at 30°C. The observation that most of the spores failed to germinate may suggest that the Cdc4p-binding activity of Pik1p may be involved in germination. More tetrad analyses should be performed to confirm this observation. Also, the tetrads should be analyzed at additional growth temperatures to rule out any temperature-sensitive effects.

The terminal phenotype of cells carrying the *pik1*^{D709A} mutation at the *pik1* chromosomal locus is different than that of cells which were deleted for the entire *pik1* gene (Park, 2007). In the latter case, most of the spores failed to germinate at 30°C suggesting a role for *pik1* in germination (Park, 2007). Some spores however, did undergo 1 or 2 rounds of division. The variability of this phenotype may be due to the varying Pik1p and PtdIns4P levels in the diploid cell before tetrad analysis and germination. It has been reported that *pik1* transcripts peak at meiosis I (Mata *et al.*, 2002). Furthermore, the loss-of-function mutant of CDP-diacylglycerol synthetase, which results in reduced levels of PtdIns, causes spores to fail germination in *S. cerevisiae* (Shen *et al.*, 1996; Shen and Dowhan, 1996). However, CDP-diacylglycerol synthetase is also required for the synthesis of other lipids such as phosphatidylserine. These results may point to the importance of PtdIns metabolites in the process of germination. Interestingly, when the spores carrying the deletion allele were grown at 19°C and 25°C, there was a higher frequency of spores that initiated division suggesting a temperature sensitive effect, compared to spores grown at 30°C (Park, 2007). This may imply that *pik1* function in germination may be a complex process. The spores

carrying the chromosomal *pik1*^{D709A} allele were not grown at temperatures other than 30°C.

Studies of a *pik1* loss-of-function mutant demonstrated that Pik1p is involved in septation, but not involved in actin ring formation (Park, 2007). A conditional loss-of-function allele of *pik1* was produced using the N-degron approach (Dohmen *et al.*, 1994; Rajagopalan *et al.*, 2004). The N-degron construct is composed of monoubiquitin followed by an arginine residue fused to a thermolabile derivative of dihydrofolate reductase (Ub-Arg(R)-dihydrofolate reductase^{ts}) that is long-lived at 25°C, but is rapidly degraded by the N-end rule pathway at 36°C (Dohmen *et al.*, 1994; Rajagopalan *et al.*, 2004). Fusion of this thermolabile protein to Pik1p was expected to render the latter conditionally short-lived at 37°C. This construct was introduced into cells deleted for the *pik1* gene to observe the terminal phenotype of this recombinant allele at the restrictive temperature.

In these cells, expression of the *pik1* N-degron construct arrested the cells at a terminal step in cytokinesis and was lethal at 37°C. F-actin ring formation and constriction appeared unaffected, but most of the arrested cells were elongated and had septa that were abnormally thick, misplaced or supernumerary. These effects were much more dramatic than, but similar to, the phenotype produced by the ectopic expression of the *pik1* kinase-dead allele (*pik1*^{D709A}) in *pik1*⁺ cells. This phenotype will be discussed in greater detail in the following section. In cells carrying only the *pik1* N-degron allele and incubated at 37°C, F-actin patches were no longer found exclusively at the cell tips, but were dispersed throughout the cells (Park, 2007). This phenotype may be related to the loss of some Pik1p-dependent Golgi functions essential for

secretory activity at the cell tips. Actin plays a key role in the endocytic process by providing the force required for vesicle invagination (Gachet and Hyams, 2005).

PtdIns4P is a precursor for the formation of PtdIns(4,5)P₂, a well known regulator of actin polymerization and depolymerisation. Some actin-binding proteins in *S. pombe*, such as profilin, may bind PtdIns(4,5)P₂ for their function explaining the actin cytoskeletal defects observed in the *pik1* loss-of-function mutant.

Transmission electron microscopy of *pik1* deletion cells carrying the *pik1* N-degion allele and grown at 37°C, demonstrated aberrations in Golgi structure (Park, 2007). This is reminiscent of *S. cerevisiae PIK1* temperature-sensitive mutants which also display Golgi defects (Audhya *et al.*, 2000). Furthermore, expression of a kinase-dead PtdIns4KIIIβ allele (D656A) in mammalian cells caused aberrations in Golgi structure (Godi *et al.*, 1999). This suggests that the discrete synthesis and localization of PtdIns4P at the Golgi may be required for Golgi structure. The mechanism for this is unclear, however, it is likely that PtdIns4P acts to localize many proteins to the Golgi required for its maintenance. Studies have shown that PtdIns4P acts as a coincidence sensor involved in facilitating interactions with other proteins at the Golgi (Strahl and Thorner, 2007; Wang *et al.*, 2007). Coincidence detection refers to the process of protein membrane recruitment through multiple relatively low affinity interactions. One thing is clear, and that is that Pik1p, because of its role at the Golgi in the production of PtdIns4P, is a key player in membrane trafficking (Godi *et al.*, 1999; Godi *et al.*, 2004; Wang *et al.*, 2007).

Since Pik1p is a Golgi associated protein (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000; Strahl *et al.*, 2005; Matsuyama *et al.*, 2006; Park, 2007), these

results suggest that Pik1p-dependent Golgi structure and/or dynamics may play an important role in septation and/or membrane expansion during cytokinesis, but not in contractile ring assembly. One pathway important for septation and membrane trafficking during cytokinesis in *S. pombe* is the septation initiation network (SIN). The SIN is a signaling cascade that triggers septation and the contraction of the contractile ring. Perhaps the SIN signals through Pik1p to properly shuttle septum building enzymes or septum cell wall material to the medial region. Also, the SIN appears to be required for membrane trafficking to the medial region since mutants of the SIN fail to form endocytic vesicles at the cell equator during cell division (Gachet and Hyams, 2005). However, it is currently unclear how the SIN acts to trigger septation, for example, which proteins it activates or deactivates. Interestingly, the negative regulators of the SIN, Byr4p and Cdc16p, a two-component GTPase activating protein (GAP) for the Spg1p GTPase, display septation defects such as supernumerary septa, similar to the *pik1* loss-of-function mutant. In order to elucidate a potential role for Pik1p in the SIN pathway, genetic analysis should be performed. It would be interesting to determine if Pik1p acted early or late in the SIN pathway by analyzing the distribution of Pik1p at the medial region in various SIN pathway mutants. Alternatively, one might look for suppression of lethal phenotypes or synthetic lethal interactions of cells double-mutant for a SIN pathway component and the *pik1* deletion strain carrying the *pik1* N-degron construct.

The exocyst is a complex important for the proper separation of cells during the later stages of cytokinesis through the exocytosis of the Eng1p and Agn1p glucanases (Wang *et al.*, 2002; Martin-Cuadrado *et al.*, 2005). When each of its components are

mutated, cells form long chains that do not separate, but the septa are apparently normal. This is a different phenotype than that of the *pik1* loss-of-function mutant since the *pik1* loss-of-function mutant demonstrates defects not only in cell separation, but in the regulation of septation since the septa are thick and supernumerary (Park, 2007). Thus, Pik1p may be involved not only in cell separation by shuttling glucanases to the medial region, but additionally in the proper regulation of the shuttling of septum cell wall building enzymes to the medial region or hydrolysis or endocytosis of septum cell wall material at the medial region.

Some cytokinesis proteins involved in septation or cell separation contain pleckstrin homology (PH) domains. PH domains of many proteins are known to bind phosphoinositides (DeMatteis *et al.*, 2005). Mid2p is a PH domain containing protein that is required for cell separation. The PH domain has not been shown to bind phosphoinositides, but is required for Mid2p function and its localization to the medial region late in cytokinesis (Berlin *et al.*, 2005; Tasto *et al.*, 2003; An *et al.*, 2004). Also, *S. pombe* septins are required for cell separation and localize to the medial region late in cytokinesis (Wu *et al.*, 2003; An *et al.*, 2004). Little is known about the septins in *S. pombe* (*spn1*, *spn2*, *spn3*, *spn4*); however, studies of the septin homologues in *S. cerevisiae* and mammals show that they bind phosphoinositides. The exocyst component, Exo70p, also binds to phosphoinositides and this function is required for exocytosis in *S. cerevisiae* (He *et al.*, 2007). Thus, Pik1p, by contributing to the regulation of phosphoinositide production, may act to recruit Mid2p, septins and the exocyst to the division site. This is consistent with the loss-of-function phenotypes of Mid2p, the septins, and exocyst subunits, which like Pik1p, fail to separate the daughter

cells into two. Mid2p, septins and the exocyst are key players in membrane trafficking at the medial region to execute cell separation since Mid2p acts to stabilize septin rings and since septin rings are required to act as positional markers for vesicles targeted to the division site by the exocyst (Berlin *et al.*, 2003; Martin-Cuadrado *et al.*, 2005).

Two additional proteins involved in membrane trafficking are required for cytokinesis and they are the AP-1 μ 1 subunit, Apm1p and the Rab11 homologue, Ypt3p. Interestingly, the loss-of-function phenotypes of these mutants match that of the Pik1p loss-of-function mutant, in that the cells have thick septa which fail to separate, thus potentially placing them in the same pathway. Perhaps Pik1p, or the PtdIns4P it produces, is involved in the regulation and/or localization of both of these proteins.

Pik1p activity may be required to terminate deposition of septum material, or to retrieve septum material by endocytosis or hydrolysis. PtdIns(4,5)P₂ accumulates at the cleavage furrow in mammalian cells (Field *et al.*, 2005) and at the medial region of dividing *S. pombe* cells (Zhang *et al.*, 2000). PtdIns(4,5)P₂ is presumably required to facilitate anchoring of the plasma membrane to components of the contractile ring (Field *et al.*, 2005). Perhaps Pik1p provides the PtdIns4P required to produce this metabolite in the cell. Alternatively, PtdIns4P may be an important signaling molecule at the medial region like it is at the Golgi. It is unlikely that Pik1p activity is required for the initial site selection and assembly of the contractile ring. First, the observed recruitment of Pik1p to the medial region occurs later than the time of F-actin ring formation (Park, 2007) and second, loss of Pik1p has no apparent effect on ring formation or constriction (Park, 2007). Finally, ectopic expression of a *pik1*^{D709A}

kinase-null allele causes defects in septation suggesting that PtdIns4P metabolites are required for cytokinesis.

5.4. Ectopic expression of *pik1^{wt}* causes dominant lethal effects in *S. pombe*

Surprisingly, ectopic expression of the wild-type *pik1* allele, which was shown to increase lipid kinase activity in cell homogenates, was lethal to *S. pombe* cells, as evidenced through colony formation assays. Through my studies, we know that Pik1p has two activities/roles: one as a PtdIns 4-kinase and one as a Cdc4p binding protein. I wished to understand if either or both of these activities contributed to the ectopic lethal phenotype of *pik1^{wt}*.

The inhibition of cell proliferation upon the ectopic expression of *pik1^{wt}* was likely due to the disruption of actin-cytoskeletal structures that are required for cell elongation and division. Instead of actin localizing to the cell poles or to the contractile ring, actin was dispersed throughout the cell as punctate staining. The product of Pik1p activity, PtdIns4P, is a precursor of PtdIns(4,5)P₂, which is involved in the formation and maintenance of actin cytoskeletal structures (Janmey and Linderg, 2004).

The ectopic expression of the *pik1^{wt}* gene under the control of the *nmt1* promoter may have altered the timing of *pik1^{wt}* expression or the abundance of the transcript, or both. The concentrations of the *pik1^{wt}* and *nmt1* transcripts have been reported to be constant during the cell cycle (Rustici *et al.*, 2004). Pik1p abundance was barely at the level of detection in our immunoassays and these low levels of Pik1p are sufficient to maintain cell growth and proliferation (Park, 2007). Thus, even low levels of expression of *pik1* would have significantly increased the cell content of a

catalytically active enzyme. Indeed, my studies have shown that after Pik1p protein levels increased, the cells had greater lipid kinase activity and higher levels of Pik1p may be expected to affect interactions with Cdc4p and other proteins. This will be elaborated on later in this section.

In *S. cerevisiae*, PtdIns4P is localized to the Golgi, nucleus, plasma membrane and endoplasmic reticulum (ER) (Di Paolo and De Camilli, 2006). It is thought that Pik1p regulates the PtdIns4P pool at the Golgi and nucleus in *S. cerevisiae*, while Stt4p regulates the PtdIns4P pool at the ER and plasma membrane (Di Paolo and De Camilli, 2006). Instead of discrete localization of Pik1p at the Golgi and medial region of the cell, ectopic expression of *pik1^{wt}* increased the cell content of a catalytically active enzyme that was distributed throughout the cells as observed through indirect immunofluorescence. Mislocalization of an active enzyme could lead to loss of spatial information if discrete formation of PtdIns4P or PtdIns4P-derived phosphoinositides are required at a certain time and place in the cell cycle.

Localized synthesis of PtdIns4P by Pik1p at the Golgi complex is presumed to be important for Golgi to plasma membrane traffic and transport from the Golgi complex to the vacuolar and prevacuolar compartments, based on studies in *S. cerevisiae* (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). Also, increasing PtdIns4P production likely leads to increased PtdIns(4,5)P₂ production at the Golgi, ER and plasma membrane and maybe even other membrane compartments in the cell such as early, late and recycling endosomes. We have compelling evidence for this. Ectopic expression of Pik1p leads to the distribution of PtdIns(4,5)P₂ all around the cell membrane and to speckles in the cytoplasm, instead of

at the cell poles and at the medial region (Desautels *et al.*, unpublished observations). In this study, PtdIns(4,5)P₂ localization was monitored by fusing the PH domain of human phospholipase C δ_1 (PLC δ_1) to eGFP. These results emphasize the importance of inositol lipid metabolism in cell growth and division in *S. pombe*, but do not point to a specific role for Pik1p, as ectopic expression of other lipid kinases or phosphatases may be expected to disrupt phosphoinositide metabolism.

As expected, ectopic expression of the kinase-dead *pik1*^{D709A} allele impaired colony formation, but surprisingly, to a lesser extent than the wild-type sequence. This allele was shown to have no lipid kinase activity and yet still bound to Cdc4p. If lipid kinase activity were the major cause of the ectopic phenotype of the wild-type sequence, then the ectopic expression of the *pik1*^{D709A} allele would be expected to be innocuous. The fact that there is still a lethal phenotype suggests that lipid kinase activity is not the only contributing factor to the *pik1*^{wt} ectopic phenotype.

After the accumulation of the Pik1p^{D709A} protein, cells showed a doubling in the number of cells with septa, and a proportion of these cells (6%) had supernumerary or thick septa, while contractile ring formation remained similar to cells carrying the vector alone. Approximately 20% of the cells were also elongated. Thus, even though the ectopic expression of the *pik1*^{D709A} allele did not impair colony formation to the same extent as the wild-type sequence, it was still acting in a dominant-negative fashion to affect septation. This is because this allele is still likely localizing to the Golgi and the medial region in these cells, competing with the wild-type enzyme, specifically disrupting its function. It is possible that the ectopic expression of the *pik1*^{D709A} allele

within the cells led to a reduction in production of PtdIns4P at discrete locations such as the Golgi or medial region of the cells.

The ectopic phenotype of the *pik1*^{D709A} allele suggests that Pik1p lipid kinase activity is required for septation, but not contractile ring assembly. In *S. cerevisiae* and mammalian cells, loss of Pik1p function (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000), and ectopic expression of a dominant-negative kinase-dead allele (Godi *et al.*, 1999), affects the integrity of Golgi structures. This is also observed in cells with a Pik1p loss-of-function mutant (Park, 2007). Loss of Pik1p lipid kinase activity may affect secretion in these cells. Formation of the septum and recruitment of enzymes necessary for cell separation require membrane traffic to and from the cell equatorial region (Wang *et al.*, 2002; Albertson *et al.*, 2005; Gachet and Hyams, 2005).

Furthermore, a reduction in PtdIns4P at the Golgi may affect the PtdIns4P concentrations at other regions of the cell from disabled vesicle trafficking. Overall, disruption of Golgi functions upon ectopic expression of a kinase-dead enzyme and/or loss of PtdIns4P synthesis at the medial region are likely responsible for the septation defect.

Ectopic expression of the kinase-dead *pik1*^{D709A} allele also had effects on actin cytoskeletal structures. Ectopic expression of the *pik1*^{D709A} allele resulted in the disruption of actin patch formation in some cells, although actin ring formation was observed at similar levels to wild-type cells carrying the vector alone. As mentioned previously in this discussion, phosphoinositides interact with actin-binding proteins, such as profilin, to affect their function (Janmey and Lindberg, 2004). The fact that not all cells were observed with actin cytoskeletal defects may have been due to differences

in gene expression as suggested by indirect immunofluorescence. In cells ectopically expressing the *pik1*^{D709A} allele and immunostained for Pik1p, differences in fluorescence intensity were observed, presumably reflecting different levels of Pik1p abundance in these cells.

There are caveats to this experiment, so the phenotype associated with the ectopic expression of the *pik1*^{D709A} allele should be interpreted with caution. Ectopic expression of the *pik1*^{D709A} allele may be competing with the other PtdIns 4-kinases and PtdIns 3-kinases in the cells disrupting their function as well. This is because *pik1*^{D709A} protein accumulation may be high enough to localize to novel compartments in the cells, non-specifically disrupting other PtdIns-dependent pathways. It should be noted however, that the phenotype associated with the ectopic expression of the *pik1*^{D709A} allele is consistent with the *pik1* loss-of-function mutant, which also shows that *pik1* is involved in septation, but not contractile ring formation.

Interestingly, the ectopic expression of the *pik1*^{R838A} allele, which has lipid kinase activity in cell homogenates, but no Cdc4p-binding activity *in vitro*, was nearly innocuous in *S. pombe* cells. This result suggests that the interaction with Cdc4p, and not increased lipid kinase activity, is causing the lethality observed with the ectopic expression of the wild-type sequence. Ectopic expression of the *pik1*^{R838A} allele in wild-type cells had very mild effects on cell proliferation and morphology compared to the ectopic expression of the *pik1* wild-type sequence and *pik1*^{D709A} sequence. Since the *pik1*^{R838A} allele has equal or greater lipid kinase activity than wild-type *pik1*, these results suggest that some function of Pik1p associated with the R838 residue, and not just increased lipid kinase activity, is a major contributor to *pik1*^{wt} ectopic lethality.

One might hypothesize that the reduction in actin rings observed with the ectopic expression of *pik1^{wt}* may have been due to sequestration of Cdc4p. However, studies with the *pik1* loss-of-function mutant reveal that this is not the case as Pik1p is not involved in contractile ring formation. Ectopic expression of *pik1^{wt}* in *cdc4^{G107S}* cells, where the Pik1p-Cdc4p interaction is presumably removed (Desautels *et al.*, 2001), corroborates the finding that the Cdc4p interaction with Pik1p may be responsible for the *pik1^{wt}* ectopic phenotype. This is because the ectopic expression of *pik1^{wt}* in *cdc4^{G107S}* cells only had a very mild effect on cell proliferation.

Ectopic expression of the *pik1^{R838A}* allele resulted in a dramatic decrease in cell length in *cdc4⁺* and *cdc4^{G107S}* cells even in repressed conditions indicating that even low levels of expression had a significant effect on cell length. Furthermore, ectopic expression of the *pik1^{R838A}* allele caused mislocalization of the expressed protein. For example, the ectopic expression of the *pik1^{R838A}* allele caused the protein to accumulate at the tips of many of the shorter *cdc4⁺* cells. This suggests that the *pik1^{R838A}* allele may act to delocalize the Pik1p protein. Since the *pik1^{R838A}* allele still contains lipid kinase activity based on the lipid kinase assays I performed, the production of PtdIns4P and resultant PtdIns(4,5)P₂ at aberrant locations in the cell may affect cell length. Also, cell elongation may require the presence of Pik1p at the cell tips, which is currently an unidentified localization of the protein. Alternatively, abnormal *pik1* localization might affect trafficking of cell elongation enzymes to the cell tips.

Cell proliferation and morphology were unaffected by the ectopic expression of the *pik1* double-mutant (*pik1^{D709A, R838A}*) in wild-type cells. Thus, two functions of Pik1p, the lipid kinase activity suppressed by the D709 mutation, and another function

suppressed by the R838 mutation, fully account for the inhibition of cell proliferation upon ectopic expression of *pik1*^{wt}.

5.5. The Pik1p-Cdc4p interaction

My studies attempted to better understand the Cdc4p-binding activity of Pik1p and its function *in vivo*. I was able to show that the full-length Pik1p protein interacted with Cdc4p by ELISA. A potential binding site for Cdc4p on Pik1p was discovered and mutants were created to evaluate the function of this interaction in *S. pombe* cells. Site-directed mutagenesis was performed to mutate a specific conserved residue in Pik1p presumed to be important for Cdc4p binding to better understand the role of this interaction. Cdc4p interacts with the IQ-motifs of type II myosins (Naqvi *et al.*, 1999; Motegi *et al.*, 2000; D'Souza *et al.*, 2001) and the primary structure of the *pik1* C-terminal region suggests the presence of an IQ-motif (I₁₂₈QKANCSVWTR₈₃₈). To investigate whether Cdc4p interacts with Pik1p via this IQ-like sequence, a conserved arginine was mutated to alanine at position 838. The *pik1*^{R838A} mutation removed the Cdc4p interaction with Pik1p in both yeast-two hybrid assays and ELISA. Thus, the *pik1* R838 residue is critical for the interaction with Cdc4p in these assay conditions. Western blot analysis of *S. cerevisiae* cell homogenates expressing the yeast two-hybrid fusion proteins, were inconclusive. In this assay, the fusion proteins were not detected with neither of the α -Pik1p, α -Gal4p-TA or α -Gal4p antisera. Thus, it is unknown whether the lack of an observed interaction between C-Pik1p^{R838A} and Cdc4p was due to protein instability. However, two lines of evidence suggest that the *pik1*^{R838A}-Gal4p-TA fusion protein was accumulating and properly folded. Firstly, the *pik1*^{R838A} allele

was able to complement *S. cerevisiae pik1-101* cells at the non-permissive temperature (Park, 2007). Secondly, the *pik1*^{R838A} protein product accumulated in *S. pombe* cells and was shown to not interact with Cdc4p in ELISAs. Furthermore, the *pik1*^{R838A} allele was shown to possess wild-type levels of lipid kinase activity suggesting that this mutant is properly folded and functional. Thus, it is highly probable that the C-*pik1*^{R838A}-Gal4p-TA fusion protein is expressed and properly folded in the *S. cerevisiae* YPB2 cells, and that the negative interaction phenotype was due to a disrupted protein-protein interaction and not protein instability.

It is possible that other Cdc4p-like proteins interact and affect PtdIns 4-kinase localization and/or activity. There are other putative IQ-motifs in the N-terminal region of the *pik1* sequence, presumably required to interact with Ncs1p, a small EF-hand protein known to bind calcium (Hamasaki-Katagiri *et al.*, 2004). The Ncs1p ortholog in *S. cerevisiae*, Frq1p, is required for optimal Pik1p activity and recruitment of Pik1p to the Golgi (Hendricks *et al.*, 1999; Strahl *et al.*, 2005). However, the absence of a significant interaction between Cdc4p and Pik1p^{R838A} suggests that it is the dominant, if not only, interaction site with Cdc4p. Also, the Cdc4p interaction with the C-terminal domain of Pik1p appears to be specific for the Cdc4p EF-hand protein since Ncs1p fails to interact with the C-terminal end of Pik1p in a yeast two-hybrid assay.

If the interaction with Cdc4p and Pik1p is the main cause of *pik1*^{wt} ectopic lethality, this effect should not be observed if *pik1*^{wt} is expressed in cells with the *cdc4*^{G107S} allele, which does not bind to Pik1p (Desautels *et al.*, 2001). In *cdc4*^{G107S} cells, ectopic expression of *pik1*^{wt} had a very limited effect on cell proliferation and morphology. The majority of cells had normal F-actin distribution and no decrease in

the number of cells with contractile rings and septa. In *cdc4*^{G107S} cells, it is the ectopic expression of the kinase-dead allele, *pik1*^{D709A}, which fully inhibits cell proliferation, in keeping with the essential role played by this enzyme.

The ectopic expression of the *pik1*^{D709A} allele was synthetically lethal in *cdc4*^{G107S} cells. This may be occurring due to several reasons. Firstly, ectopic expression of the *pik1*^{D709A} allele may be disrupting a function essential for *cdc4*^{G107S} viability at 25°C. This function may be buffered by the Cdc4p-Pik1p interaction in wild-type cells, since the ectopic expression of the *pik1*^{D709A} allele in these cells is not as lethal as in *cdc4*^{G107S} cells. Secondly, the synthetic lethality observed may be a non-specific and additive effect of *pik1*^{D709A} ectopic expression. The *cdc4*^{G107S} cells have defects in cell proliferation and septation at 25°C and the ectopic expression of the *pik1*^{D709A} allele may exacerbate this growth defect through an alternate means. Finally, the cell proliferation defect upon the ectopic expression of the *pik1*^{D709A} allele in *cdc4*^{G107S} cells may indicate that this allele may still have Cdc4p binding activity. However, this is unlikely to be the case, as the ectopic expression of the wild-type sequence was nearly innocuous in a *cdc4*^{G107S} background where presumably, there is no Cdc4p-Pik1p interaction. No significant changes in contractile ring formation and septation were observed in the cells ectopically expressing the *pik1*^{D709A} sequence. Thus, it is unclear as to why they are failing to proliferate. One possibility is that Golgi structures, and therefore secretion, may be affected without significantly altering septation or contractile ring formation in these cells.

The ectopic expression of the *pik1*^{D709A, R838A} allele in *cdc4*^{G107S} cells caused a cell proliferation defect, but not as great as the single *pik1*^{D709A} mutant. It is unclear

why this would be so; however, this allele was also shown not to have lipid kinase activity, and thus, it is possible that it is also disrupting a pathway essential for *cdc4*^{G107S} cell viability at 25°C. The observation that the ectopic expression of this allele is not as lethal as the single *pik1*^{D709A} mutant, may suggest that another protein aside from Cdc4p may require the R838 residue for interaction. As expected, the ectopic expression of the *pik1*^{R838A} allele was innocuous in *cdc4*^{G107S} cells since this allele had no Cdc4p binding activity.

Cells carrying a genomically integrated *pik1*^{R838A} allele were able to proliferate, suggesting that the *pik1* R838 residue is not essential for haploid cell division. Apparently, there are Cdc4p modifications which are not essential for cytokinesis, since Cdc4p was shown to be phosphorylated at serines 2 or 6, but not both, and these phosphorylation events were not essential for cytokinesis or for its interaction with Myo2p (McCollum *et al.*, 1999a). Furthermore, if Cdc4p exists in a complex with Pik1p and other proteins, its function may be genetically redundant. It is interesting to note, that the essential light chain of scallop myosin was shown to bind calcium in the presence of the regulatory light chain and myosin interactions (Xie *et al.*, 1994). Thus, it is plausible that Cdc4p may bind to calcium upon the interaction with Pik1p. If this is the case, then the *pik1*^{R838A} allele may display proliferation abnormalities in the presence of high calcium levels. Finally, it is unknown whether the *pik1*^{R838A} allele may still interact with Cdc4p in these cells.

Studies with purified Cdc4p and a peptide of the Pik1p IQ-like sequence demonstrated that the affinity of the interaction was very low ($k_d \sim 1$ mM) (Escobar-

Cabrera, unpublished results). If this is the case, then the interaction would be highly dependent on localized concentrations of both proteins in the cell. This finding also suggests that the Pik1p interaction with Cdc4p may be very transient.

The IQ-motif at the C-terminal end of Pik1p may be present in other organisms such as *S. cerevisiae*, *C. albicans*, *C. elegans*, *D. melanogaster*, *X. laevis*, bovine, and human species (Figure 4.1). The first position of the IQxxxRGxxxR consensus sequence is somewhat variable. It is generally Ile, Leu or Val, but Met, Phe and Thr have also been observed at this position in the IQ-motifs of myosins (Bahler and Rhoads, 2002). This variability is observed within the IQ-like motifs of Pik1p across species. Furthermore, position 11 of the consensus sequence is typically occupied by an Arg or Lys, and this is also consistently observed within the IQ-like motifs of *pik1* orthologues. The central residues are known to be less conserved (Bahler and Rhoads, 2002). These characteristics of IQ-motifs match the IQ-like sequence identified in Pik1p and the conservation of some of the key residues suggest that this interaction may be present in other organisms.

5.6. Cdc4p directly or indirectly affects PtdIns metabolism

Surprisingly, no increase in lipid kinase activity was observed upon the ectopic expression of the *pik1*^{wt} allele in *cdc4*^{G107S} cells. There was a modest increase in lipid kinase activity with the ectopic expression of the *pik1*^{R838A} allele in *cdc4*^{G107S}, although this allele accumulated in protein to higher levels than the other alleles in *cdc4*^{G107S} cells. As the *pik1*^{R838A} allele has equal or greater lipid kinase activity than the wild-type enzyme, but does not interact with Cdc4p *in vitro*, these results suggest that the Cdc4p

interaction with Pik1p at the C-terminal IQ motif has limited if any effect on Pik1p lipid kinase activity. Reduced accumulation of the ectopically expressed proteins in the *cdc4* conditional strains relative to the wild-type strain at the permissive temperature of 25°C may have been the cause for the lack of an observed effect on lipid kinase activity. Although, western blot analysis shows that the Pik1p protein and its alleles accumulate to levels similar to that in wild-type cells in the *cdc4*^{G107S} cells. Alternatively, inhibition of Pik1p lipid kinase activity not involving the R838 residue and/or concurrent activation of some lipid phosphatase in the crude extracts could explain why no apparent increase in lipid kinase activity was observed upon the ectopic expression of the *pik1*^{wt} allele in *cdc4*^{G107S} cells.

It is likely that the lack of an observed increase in lipid kinase activity with the *pik1* wild-type and *pik1*^{R838A} sequences in *cdc4*^{G107S} cells is due to indirect effects. For example, Cdc4p may be involved in a protein complex with Pik1p and may require the presence of other proteins required for Pik1p activation. This is supported by the observation that in lipid kinase assays with purified Cdc4p protein, no effect on *pik1* lipid kinase activity was observed. Here, various concentrations of Cdc4p purified protein (0.1-10 mM Cdc4p) were added to aliquots of 0.4 µg of cell homogenates carrying expressed Pik1p protein. No effect on the lipid kinase activity in these cell homogenates were observed (data not shown). The lack of an effect observed with purified Cdc4p may be due to Cdc4p protein levels already in the extract. For example, all the expressed Pik1p in the cell homogenate may be saturated with Cdc4p since protein levels of Cdc4p are readily detectable without over expression in *S. pombe* cells.

5.7. Do Cdc4p and Pik1p co-localize?

Both *cdc4* and *pik1* expression levels through the cell cycle have been studied. The transcript levels of *cdc4* increase towards the final stages of mitosis, whereas *pik1* expression remains constant (Rustici *et al.*, 2004). It is interesting that *cdc4* transcript levels increase during the late stages of mitosis, controlled by the Ace2p transcription factor, which induces the expression of many genes involved in cell separation (Rustici *et al.*, 2004). This points to a potential role for Cdc4p in the late stages of cytokinesis apart from its firmly established role in the assembly of the contractile ring with Myo2p and Rng2p; an early mitotic event (Figure 5.1). This is corroborated by the finding that at 25°C, the septation index of *cdc4*^{G107S} cells is elevated while contractile ring formation remained normal. At the permissive temperature, *cdc4*^{G107S} cells showed a septation index of 18%. Wild-type cells with the vector alone at 30°C show that approximately 10% of the cell population have a septum. These experiments were conducted at different temperatures, thus it is uncertain if wild-type cells have a higher septation index at 25°C. However, this is unlikely to be the case. It still remains to be concluded if Cdc4p and Pik1p colocalize at the medial region or elsewhere in the cell. Cdc4p localizes to the medial region early in cytokinesis presumably to assemble and regulate the contractile ring with the type II myosin Myo2p and the IQGAP Rng2p (McCollum *et al.*, 1995; D'Souza *et al.*, 2001). Pik1p however, localizes to the medial region shortly after septation reflecting a potential role in septation. Thus, it is uncertain whether there is overlap in the localization of these two proteins at the medial region during cytokinesis or whether their interaction occurs elsewhere in the cell.

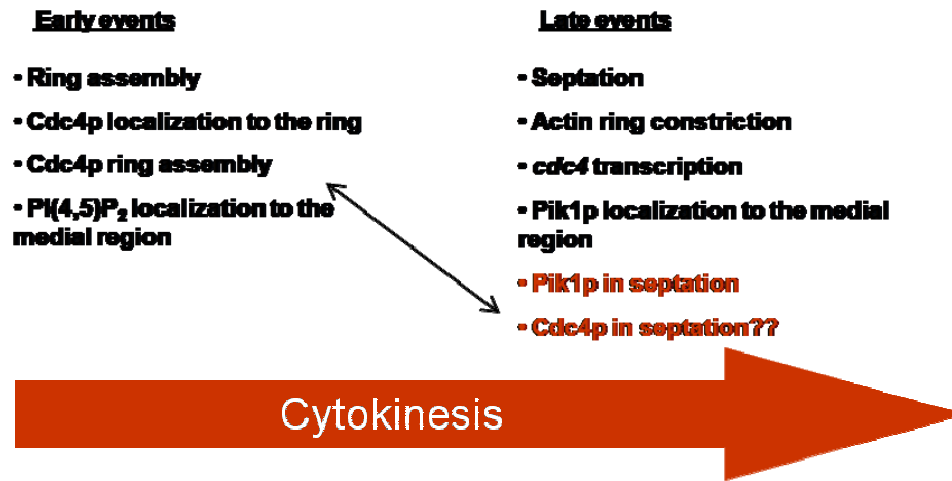


Figure 5.1: The localization and roles of Cdc4p and Pik1p in cytokinesis in *S. pombe*.

The large arrow at the bottom illustrates the progression of cytokinesis from the early stages of contractile ring formation to the late stages where contraction and septation ensues. Cdc4p localizes to the medial region early in cytokinesis whereas Pik1p localizes to the medial region late in cytokinesis. Thus, it is unclear as to where these two proteins localize. Further studies need to be performed to establish the localization of this complex *in vivo*. The double-ended arrow at the center of the figure illustrates a potential functional link between Cdc4p and Pik1p. As described in the text, there is a potential role for Cdc4p in septation. The potential association between Cdc4p and Pik1p functions is highlighted in red in the late events column. Since Pik1p localizes to the medial region late in cytokinesis, it is unclear whether Pik1p is required to generate the PtdIns(4,5)P₂ pool at the medial region. Perhaps another PtdIns4P producing enzymes is required to generate this pool, or perhaps PtdIns4P is generated at the Golgi by Pik1p and later shuttled to the medial region. PtdIns(4,5)P₂ has been shown to be required for cytokinesis in *Drosophila* and crane fly spermatocytes.

Another possibility is that Cdc4p may localize with Pik1p at the Golgi or in the cytoplasm. Perhaps the increase in *cdc4* transcripts at anaphase increases the Cdc4p protein concentration in the cytoplasm and the Golgi. This is consistent with studies of Cdc4p concentrations in the cell, which show that Cdc4p concentrations are 10-fold higher than that of Myo2p and Rlc1p suggesting that Cdc4p may exist in complexes with other proteins in different compartments of the cell (Wu and Pollard, 2005). One method for studying the cellular localization of the Pik1p-Cdc4p interaction would be FRET with split YFP (Bracha-Drori *et al.*, 2004). In this method, two proteins are independently tagged with complementary portions of YFP. Upon a protein-protein interaction, YFP is reconstituted and yellow fluorescence is observed.

5.8. Future studies

Some outstanding issues arise from these studies. The ectopic expression of the *pik1^{wt}* allele caused the majority of the cells to arrest as mononucleated cells from the pleiotropic effects caused by the deregulation of protein-protein interactions and deregulation of lipid kinase activity. It would be interesting to determine if these cells indeed arrested at a certain point in the cell cycle. Also, mutation of the R838 residue appeared to bypass this cell cycle arrest allowing the cells to proliferate. FACs analysis could help in determining which stage of the cell cycle arrest occurs in these cells.

It would also be interesting to determine if other Pik1p protein-protein interactions could contribute to *pik1^{wt}* ectopic lethality. Mutation of a residue in the N-terminal domain of Pik1p, thought to be important for the interaction with Ncs1p was also performed (R118 to A, data not shown). The R118 residue resided in a region

similar in sequence to IQ-motifs and homologous to a region required for the interaction between Pik1p and Frq1p in *S. cerevisiae*. Mutation of this residue removed the *pik1^{wt}* ectopic phenotype. Interestingly, this allele was shown not to have lipid kinase activity consistent with studies in *S. cerevisiae* which showed that Frq1p acted to activate Pik1p lipid kinase activity. It would be interesting to replace this allele with the wild-type *pik1* genomic copy to see if this interaction was essential for haploid growth. Yeast two-hybrid analysis did not demonstrate whether the interaction between Ncs1p and Pik1p was abrogated by the mutation of this residue. Further interaction studies would be required to determine if *pik1^{wt}* ectopic lethality was due to an additional protein-protein interaction with Ncs1p. See Appendix for additional plasmids used in this study.

Ectopic expression of a kinase-dead PtdIns4KIII β allele in mammalian cells caused a dominant-negative effect by disrupting Golgi structures, and the *pik1* loss-of-function mutant produced the same effect (Godi *et al.*, 1999; Park, 2007). Transmission electron microscopy of cells ectopically expressing the lipid kinase-dead *pik1^{D709A}* mutant should be analysed for similar defects in Golgi structure. Here, the lack of the recruitment of Golgi proteins through the reduction in PtdIns4P at the Golgi may explain the effects in Golgi structure. It would be interesting to determine the contribution of lipid kinase activity specifically to this defect.

The lipid kinase activity assays performed in these studies used crude homogenates of cells carrying the many PtdIns metabolizing proteins expressed in the vegetative life cycle during log phase growth. Thus, the direct effects from the ectopic expression of the *pik1^{wt}* allele are masked by the presence of these other proteins. Since

expression of the Pik1p protein in BL21(DE3) cells yielded an insoluble product, immunoprecipitation of the protein with an antibody generated against Pik1p would be ideal. However, the available antibodies generated towards Pik1p utilized the C-terminal domain which includes the catalytic domain. It is plausible that this antibody may interfere with Pik1p lipid kinase activity. Thus, it would be beneficial to generate antibodies directed against the N-terminal domain of Pik1p to bypass this potential problem. It is interesting to note, that antibodies to Pik1p in *S. cerevisiae* were generated against the N-terminal domain which detected its localization to the nucleus (Walch-Solimena and Novick, 1999). Tagging the *pik1* allele with two EGFP molecules did not clearly reveal a nuclear localization of the protein. Perhaps these antibodies may reveal this localization in *S. pombe* cells.

Finally, further studies in determining the biological function of the Pik1p-Cdc4p interaction should be performed. Firstly, studies to determine whether the *pik1*^{R838A} cells have a temperature-sensitive defect should be determined. Secondly, the *pik1*^{R838A} cells should be probed for synthetic lethal interactions which may reveal sources for genetic redundancy, or analyzed for their conditional growth in the presence of certain compounds, such as CaCl₂. Unfortunately, there is no set of viable haploid deletion mutants for the fission yeast genome as exists in *S. cerevisiae*. In *S. cerevisiae*, one can screen a large number of viable deletion strains (~4700) for synthetic lethal interactions, termed systematic genetic analysis (SGA) (Tong *et al.*, 2001). However, in *in lieu* of these useful gene deletion sets, synthetic lethal screens can be performed.

Synthetic lethal screens are powerful tools for identifying additional genes in a pathway (Forsburg, 2001). For a synthetic lethal screen, an *S. pombe* strain which has

the chromosomal *ura4-D18*, *ade6-M210/M216* and *pik1^{R838A}* mutants are transformed with a plasmid that contains the wild-type *pik1* gene with the *ura4* and *ade6* markers. These cells are then mutagenized with EMS or nitrosoguanidine. The resultant colonies are replica plated onto plates with low adenine and 5-FOA (5'-fluoro-orotic acid). The pink colonies which grow on 5-FOA can be discarded since they likely represent an irrelevant mutation in a second gene which does not cause the cells to fail colony formation in the presence of the *pik1^{R838A}* chromosomal mutation and are therefore not synthetically lethal. The colonies that fail to form in the presence of 5-FOA, however, likely represent either a loss-of-function mutation in *pik1* or a synthetic lethal mutation in a second gene *syn1-1* (arbitrary name). The second gene can be identified by complementation if it is recessive. For example, if a diploid strain *pik1^{R838A}/pik1^{R838A} syn1-1/syn1* is viable, then the synthetic lethal mutation *syn1-1* is recessive. If this is the case, then one can clone the *syn1* gene by keeping the *pik1^{R838A}syn1* cells alive with the original *pik1* plasmid. The cells can then be transformed with a plasmid library containing wild-type genes and no *ura4* marker. Upon plating on 5-FOA plates, the parent plasmid carrying the *pik1* sequence and *ura4* marker is driven out, and the cells carrying either the wild-type *pik1* gene or *syn1* gene form colonies. Alternatively, one could perform a synthetic dosage lethal screen with a library of wild-type genes under the control of the *nmt1* promoter. The cells that do not form colonies when replica plated onto plates lacking thiamine could uncover genes which are synthetically lethal with *pik1^{R838A}* in a dosage-dependent manner. The synthetic dosage lethal genes could be easily identified by plasmid recovery and sequencing.

However, there are several candidate proteins which may yield synthetic lethal interactions with *pik1*^{R838A} cells. *S. cerevisiae* SGA analysis of a *PIK1* temperature-sensitive mutant, *pik1-139*, yielded synthetic lethal interactions with the proteins Ypt31p, Drs2p, Trs33p and Kre11p; proteins involved in Golgi anterograde transport to the plasma membrane (Sciorra *et al.*, 2005). Ypt31p is a Rab GTPase involved in secretory transport by regulating vesicle formation at the *trans*-Golgi (Benli *et al.*, 1996; Jedd *et al.*, 1997). Drs2p is a Golgi associated, integral membrane aminophospholipid translocase required for making clathrin-coated exocytic vesicles (Chen *et al.*, 1999; Gall *et al.*, 2002). Trs33p and Kre11p encode two non-essential subunits of the TRAPP^{II} complex, a large Golgi associated complex consisting of 10 subunits (Sacher *et al.*, 2001). The TRAPP^{II} complex is a GTP exchange factor (GEF) for the Ypt31p GTPase (Sciorra *et al.*, 2005).

Interestingly, the genetic interaction observed between *S. cerevisiae* *PIK1* and *YPT31* appears to be conserved since a direct interaction between PtdIns4KIII β and the *YPT31* homologue Rab11 was observed in mammalian cells (de Graaf *et al.*, 2004). The same module may be conserved in *S. pombe* and it is plausible that Cdc4p may play a role in this complex. The Cdc4p homologue in *S. cerevisiae* may not have been identified in the SGA screen since *mlc1* is an essential gene. A search for homologues of these proteins in *S. pombe* indicate that all proteins except for Kre11p are conserved. The Ypt31p homologue in *S. pombe* is Ypt3p, and interestingly it is essential for haploid growth, unlike in budding yeast where it is genetically redundant with Ypt32p (Benli *et al.*, 1996; Jedd *et al.*, 1997). There is one available temperature-sensitive mutant of Ypt3p (*ypt3-i5*) with which functional studies could be performed (Cheng *et*

al., 2002). Interestingly, the *ypt3-i5* mutant displays a remarkably similar phenotype to the *pik1* loss-of-function mutant suggesting that this module may be conserved in *S. pombe*. The Drs2p and Trs33p proteins have homologues in *S. pombe*, but have not been experimentally characterized. The loci correspond to SPBC887.12 and SPAC13G6.05c, respectively.

Overall, these results suggest an essential role for Pik1p lipid kinase activity in cell separation. There is an interaction between Pik1p and Cdc4p whose functional nature is still uncertain and is not essential for cell viability in that cells containing the R838A mutation at the genomic locus of *pik1* are still able to divide and form colonies. However, this does not mean that the Cdc4p interaction with Pik1p is unimportant. Cdc4p may help in the recruitment of Pik1p to an unknown compartment in the cells rather than directly modulate the enzyme activity.

Chapter 6: Bibliography

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Appendix

Additional mutants used in this study

Organism	Plasmid	Strain Number	Source
<i>E. coli</i> XL1-Blue MRF'	pREP1- <i>pikI</i> ^{R118A}	1445	This study
<i>E. coli</i> XL1-Blue MRF'	pREP1- <i>pikI</i> ^{R118,D709A}	1444	This study
<i>E. coli</i> XL1-Blue MRF'	pREP1- <i>pikI</i> ^{R118A,R838A}	1462	This study
<i>E. coli</i> XL1-Blue MRF'	pREP1- <i>pikI</i> ^{R118A,D709A,R838A}	1443	This study